THE ROLE OF HUMORAL FACTORS IN CELLULAR RESISTANCE

by

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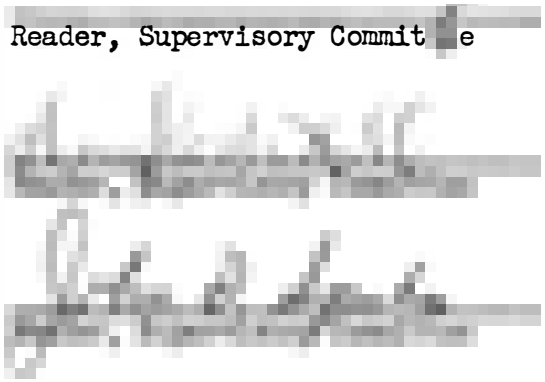

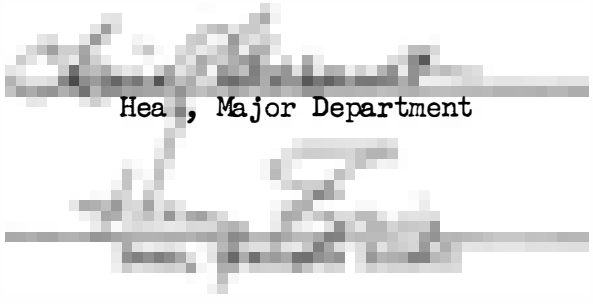

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INTRODUCTION

At present there are two major concepts regarding the mechanism of acquired resistance to infectious diseases. The first, and most popularly known, is the humoral theory as first put in general form by Ehrlich in 1904 (1906). Here it was suggested that substances (antibodies) are formed which enter the circulatory system of a host and are capable of deterring the pathogenic properties of infectious agents. The second theory was set forth by Elie Metchnikoff in 1883 (Metchnikoff, 1905) and its importance has become increasingly evident. It was proposed by this investigator that phagocytic cells, for which he coined the functional terms "microphages" (for polymorphonuclear phagocytes) and "macrophages" (for mononuclear phagocytes), were all-important in both natural and acquired immunity. Increased resistance in acquired immunity was brought about, according to Metchnikoff (1905), by an increase in the protective activity of these cells when the host had been previously exposed to an infectious agent.

Subsequent investigations into these areas of immunity have shown that it is probable that the humoral and cellular mechanisms are complementary in acquired resistance; however, the degree of importance of one over the other may vary with the type of infection. For example, it has been reported that serum from immune animals does not play a significant role in certain chronic diseases, such as tuberculosis (Lurie, 1942; Bloch, 1961) and histoplasmosis (Hill and Marcus, 1959; Miya and Marcus, 1961), while certain phagocytic cells appear to acquire an increased capacity to combat these types of infectious agents.

Although both theories of immunity were enunciated during the same era, research into cellular immunity has been comparatively tedious and difficult. A major problem in this regard has been to separate the activity of cells from that of humoral factors accompanying the immune process in order that any changes in cellular activities might be made evident. In fact, it has only been within the last twenty years that progress has been made in separating these two phenomena such that cellular immunity could be studied in the relative absence of antibody.

A second major problem involved in elucidating the cellular mechanism has been the lack of procedures to quantitate the phagocytic and cytopeptic processes of the cells. This is especially important in attempting to study the effects of humoral factors such as antibody, whole complement, complement fractions, and properdin on the cells in question where small but significant differences in cellular activity may not become apparent unless quantitative procedures are employed.

Recently, Hill and Marcus (1960) devised an in vitro system combining tissue culture methods and radioisotope labeling which appears to overcome these major difficulties. That is, phagocytes may be studied apart from or in the presence of various added humoral factors and the procedure allows one to distinguish between and to quantitate the cellular processes of phagocytosis and intracellular digestion (cytopepsis).

The subject of this thesis includes an in vitro study of cellular immunity against Histoplasma capsulatum employing a modification of the procedure of Hill and Marcus (1960). The objectives of the work to be presented include: (1) an attempt to further explore the theory of

Metchnikoff, in a quantitative fashion; that is, that immunization enhances cellular resistance as it applies to histoplasmosis; (2) the delineation of the effects of various humoral factors on the ingestive (phagocytic) and digestive (cytopeptic) processes of phagocytes derived from normal and immune animals; (3) an attempt to correlate the findings outlined here with known immunologic principles and experimental evidence.

REVIEW OF LITERATURE

I. Cells Concerned with Phagocytosis

There are a variety of morphologically distinguishable cells distributed throughout the mammalian body which among their functions include concern with the defense of the host against unwanted particulate matter. These are collectively known as "phagocytic cells" due to their ability to ingest and attempt to rid the host of this material. The aggregation of cells having this function is termed the "phagocytic system," and include (1) the polymorphonuclear and mononuclear leukocytes of the circulatory system. Lymphocytes, eosinophils and basophils have been included by different investigators. (2) The reticuloendothelial system forms a second major entity of the phagocytic system.

A. Phagocytic Cells of the Circulatory System.

1. The polymorphonuclear leukocytes. In the event that a host organism is subjected to an acute inflammatory stimulus, one of the primary phagocytic cells that migrates to the site is the polymorphonuclear leukocyte (Fowler, 1949; Suter, 1956; Florey, 1959), also commonly known as the PMN, neutrophil, or microphage; the latter term as previously mentioned, was coined by Metchnikoff (1905) to designate these cells, as well as the eosinophils.

The polymorphonuclear leukocyte originates extravascularly in the bone marrow (Fowler, 1949). Apparently, these cells which are attracted to an area of acute inflammation (positive chemotaxis) due to the release

of some substance from the injured tissue (McCutcheon, 1946; Fowler, 1949) or from bacteria (McCutcheon, 1946, Harris, 1954), subsequently ingest foreign material. However, these cells are not phagocytic toward all bacteria, and do not ingest worn-out fragments of tissue (Fowler, 1949); this latter function is carried out by other phagocytes, such as lymphocytes (Smith and Lewis, 1958) and monocytes (Wintrobe, 1951).

The neutrophil plays an important role in preventing the access of bacteria into the tissue in the absence of inflammation by migrating to the mucous membrane surface where potentially pathogenic organisms may be present (Fowler, 1949).

The PMN's provide from 54 to 62% of the total white blood cell content in the circulatory system of normal adult humans and different concentrations in other species (Wintrobe, 1951).

2. The eosinophil. The eosinophils (eosinophilic leukocytes or microphages) comprise from 1 to 3% of the circulating leukocytes of humans (Wintrobe, 1951). These cells appear to have phagocytic powers (M'Cririck, 1911-12; Strumia and Boerner, 1937), although relatively little is known concerning their activity because of the difficulty in obtaining a large number of cells from different sources (Mudd et al., 1934).

These white cells arise from the extravascular fixed reticular tissue, similar to the polymorphonuclear leukocyte (Fowler, 1949), and exhibit similar chemotactic powers (Harris, 1954).

3. The basophils. Some evidence has been presented to show that the basophils are phagocytic, although such information is scanty. Ringoen (1922-23) observed the phagocytosis of polymorphonuclear leukocytes

by these cells, as did Strumia and Boerner (1937) using staphylococci. In both cases, however, phagocytic activity was less than that shown by PMN's.

The opinion is also held that basophils are not phagocytic at all, although they are ameboid (Downey, 1938). Evidence suggests that the major function of the "mast cell" is delivery of anticoagulant to an area of inflammation through secretion of heparin containing granules, thus facilitating absorption or preventing clotting of blood and lymph in obstructed tissue (Ehrich, 1948).

Undoubtedly, as in the case of the eosinophils, difficulty in determining the true phagocytic nature of these cells is due to the relatively low numbers occurring in the blood stream, consisting of only 0 to 0.75% of the total circulating white cell population (Wintrobe, 1951).

4. The lymphocyte. There is disagreement in the literature concerning whether the lymphocyte should be considered a phagocytic cell. Wright and Dodd (1955), for example, state that most investigators agree that the mature lymphocyte is not phagocytic. Others observe that it is neither phagocytic nor bactericidal (Downey, 1938), and that it does not play any role in acute inflammation so far as is known (Florey, 1959). These cells do, however, aid in walling off infection, and may be involved in the production of antitoxins, or in removal or absorption of toxin (Downey, 1938). Thus, even though these white cells may be present in large numbers in areas of chronic inflammation their function is largely unknown (Downey, 1938; Florey, 1959).

On the other hand, Rebuck and Crowley (1955) have reviewed a series of papers covering the first half of this century, many of which give evidence that lymphocytes have phagocytic capacities and are able also to transform into macrophages, which are a highly phagocytic group of cells.

It is interesting to note that Metchnikoff (1905), who derived the term "macrophage," applied it in part to the large lymphocyte, which he considered to be phagocytic, as contrasted to the small lymphocyte which was considered not to be phagocytic and was not included in his terminology as a macrophage.

In their own study, Rebuck and Crowley (1955) reported observations of lymphocyte transformation to macrophages. They found that the lymphocytes migrated to an area of insult, with subsequent macrophage formation in from 16 to 20 hours after the stimulus. More recently, Rebuck and LoGrippo (1961) have confirmed this finding. However, Mudd, McCutcheon and Lucké (1934) expressed the opinion that whether lymphocytes are phagocytic is complicated by the belief of many competent investigators that they may transform into cells universally accepted as phagocytic, although Rebuck and Crowley (1955) pointed out that they were readily able to distinguish macrophages arising from lymphocytes from those arising from monocytes by the distinct differences in morphology of nuclei of the two cells after transformation.

Apparently, lymphocytes are not endowed with the property of chemotropism (Dixon and McCutcheon, 1935), even though as previously mentioned Rebuck and Crowley (1955) did observe lymphocyte migration to an area of insult. In any case, the cells are capable of ameboid motion (Downey, 1938).

B. The Reticuloendothelial System.

The second major divison of cells involved in the cellular defense of the body is that known as the reticuloendothelial system (RES). Included in this complex network of phagocytic cells are the monocytes of the circulatory system; sessile cells within the sinusoids of the liver, spleen, bone marrow, lymph nodes, adrenal cortex and anterior lobe of the pituitary; sessile or resting-wandering cells of the connective tissue in various organs (histiocytes, clasmatocytes, or macrophages); and microglial cells (Suter, 1956). Free macrophages of the lung (alveolar phagocytes) are also included here, however, their source is uncertain. That is, they may arise from "septal cells" of the alveoli, or may be of hematogenous origin (Maximow and Bloom, 1957).

1. The monocyte. The monocyte probably originates from the reticular cells of the RES, and provides from 3 to 7% of the circulating leukocytes in normal blood (Wintrobe, 1951). The function of this cell includes multiplication, phagocytosis, synthesis and elaboration of systems required to develop into the macrophage (Tomkins, 1955). This latter function is said to take place under conditions of inflammation (Downey, 1938), when the monocyte undergoes hypertrophy (Maximow, 1927). Others have found that the function of the macrophage and the monocyte are indistinguishable (Downey, 1938), and that they are similar to macrophages arising from the tissue (Florey, 1959). Subsequent passage of time may result in their transformation from macrophage to histiocyte (Florey, 1959). Furthermore, under conditions of tissue culture, the monocyte has been found to develop into fibroblasts (Downey, 1938).

2. The histiocyte. Sessile histiocytes, or resting-wandering cells, are branched polymorphous cells of the loose connective tissue. Under normal conditions some of the cells withdraw their processes and start to migrate, becoming free or mobile histiocytes. This phenomenon is greatly stimulated by slight irritation (Downey, 1938). Both the fixed and the mobile cells play a predominant role among cells engaged in the defense reactions. In addition to phagocytosis and intracellular digestion of foreign material, they appear to be one source of substances (antibody) in the blood which indicate the body response to infection (Jaffe, 1931).

3. Microglia and the alveolar phagocytes. The microglial cells are a particular type of phagocytic cell located in the central nervous system (Florey, 1959). These cells are classified within the RES because of their similarity of origin and ingestive properties to others within the system (Berry and Spies, 1949).

Alveolar phagocytes are highly phagocytic and behave similarly to histiocytes. They may originate from pre-existing "septal" cells of the alveolar wall (Maximow and Bloom, 1957) or possibly from blood monocytes or sinusoid phagocytic cells (Florey, 1959).

In the following table an effort has been made to summarize the morphological types and functional activities of the phagocytic cells in the light of current theory.

TABLE 1.

Phagocytic cells and their relative ability to ingest particulate matter

(Adapted from Zinsser, Enders and Fothergill, 1940).

MICROPHAGES Granulocytic cells of the circulating blood			MACROPHAGES Mononuclear cells, including fixed and wandering cells of the tissues and of the blood				
Neutrophils polymorpho-nuclear leukocytes (most active)	Eosinophils	Basophils (Conflict-ing reports as to activity)	Fixed endo-thelial cells of the blood and lymph sinuses of certain organs (most active)	Histiocytes of the con-nective tissue (very active)	Alveolar phagocytic cells of the lung (very active under cer-tain con-ditions)	Splenic pulp cells and blood mono-cytes (moder-ately active)	Reticular cells of the spleen, liver, and lymph nodes (least active)

II. Cellular Immunity

The concept that phagocytic cells could be involved in acquired immunity occurred to Metchnikoff (1905) through his work with lower animals such as the daphnia (a small, transparent, water crustacean), sea anemones, and transparent flat worms. In subsequent studies with higher animals further evidence was obtained to suggest validity of his theory. For example, frogs that had been immunized with Bacillus pyocyaneus (Pseudomonas aeruginosa) became less susceptible to infection to this organism because, it was thought, of increased activity of phagocytic cells. Furthermore, in guinea pigs vaccinated with vibrio organisms, then treated with opium to immobilize the phagocytes, resistance was reduced on subsequent challenge. In other experiments in which rabbits were immunized against the bacillus causing swine erysipelas and mouse septicemia, the phagocytes were observed to dispose of the organisms through intracellular digestion more rapidly than those from normal animals. Therefore, according to Metchnikoff, acquired immunity was due at least in part to "perfecting of the phagocytic and digestive powers of the leukocytes--a general superactivity and adaption of the phagocytic reaction...."

However, one criticism which might be made of these results is the lack of adequate partition of cellular and humoral factors in immunity, a problem, as mentioned previously, still existing today. However, one experiment described by Metchnikoff (1905) appears to give qualitative evidence favoring his hypothesis of increased intracellular destructive

capacity in the absence of antibody. This experiment was carried out by immunizing dogs against gelatin and then testing the leukocytic extracts from these and normal animals for ability to inhibit gel formation in vitro. Under the circumstances employed, it was found that the extract from "immune" cells was 5 times more effective in inhibiting gel formation than that from normal cells. On the other hand, in vitro experiments employing an experimental design which has served as a model for many later investigators, Denys and LeClef (1895) were unable to show any differences in ingestion or digestion of streptococci by phagocytes from normal and immune animals when suspended in normal or immune sera. It is not unlikely, though, that in this study technical difficulties may have masked any difference that might have been present.

Following the studies of Metchnikoff many investigators have attempted to verify and extend his findings, but not all have been successful. As an example, Robertson and van Sant (1939) found that macrophages from immune dogs were devoid of anti-pneumococcal activity in vitro in the absence of opsonic fluids. Furthermore, they were unable to show any significant differences in the rates of phagocytosis between normal and "immune" cells. Similarly, in a study by Cannon, Sullivan and Neckermann (1932) staphylococci and paratyphoid organisms were cleared more rapidly from the blood stream of immunized animals than that of non-immunized controls. Microscopic examination of the liver and spleen of these animals showed increased numbers of bacteria and increased bacterial disintegration in the immunized group. These authors, as did Robertson and van Sant (1939), attributed the observed results to specific antibody rather than any enhanced cellular capacity.

In contrast to these studies emphasizing the role of antibody, Sullivan, Neckermann and Cannon (1934) published results favoring cellular immunity. Their study is suggestive of that carried out by these workers (Cannon et al., 1932) previously where bacteria were found to be cleared from the blood stream of immunized rabbits more rapidly than from normal animals. In addition, the liver and spleen of the immune animals killed the organisms at a higher rate than those of normal animals leading these authors to conclude that immunization resulted in elevation of the functional state of the macrophage system. However, in this study, as in others previously mentioned, the participation of antibody was not defined and it is quite evident that the true role of phagocytic cells in acquired immunity remained to be defined.

An indirect procedure carried out by Rich and McKee in 1934 gives further insight into cellular immunity, moreso perhaps, than other studies carried out prior to 1940. In these experiments rabbits were immunized against pneumococci; then the animals, along with a normal group, were treated with benzene to produce a leucopenia. Subsequent intradermal challenge of the nonimmunized leucopenic animals with the virulent organisms led to a rapid spread of the bacteria within the tissues and blood, with death ensuing within 24 hours. This is compared to less rapid spread but death within 48 hours in nonleucopenic normal rabbits. Immune leucopenic animals given the same intradermal challenge showed initial localization of the organisms, which was attributed to the bacteria "sticking" within the area due to the presence of antibody. These eventually grew out, however, and produced a septicemia and death within 4 to

5 days. This was contrasted to an immune nonleucopenic group of animals subjected to a similar challenge which survived without ill effect. Thus, it is evident that cellular factors did play a significant role in this particular study, but here again certain factors interfere with analyzing the experimental results, not least of which is the fact that a complete leucopenia was not obtained.

Beginning in the early 1940's significant inroads began to be made into exposing the role of acquired cellular immunity. Because of the importance of technical factors it appears justified to summarize the following experimental work on a procedural basis so that some common comparisons may be made.

In the following portion of this review only certain points will be emphasized as these apply to the study involved. For general reviews covering phagocytosis the reader is referred to Mudd, McCutcheon and Lucké (1934), Taliaffero (1949), Berry and Spies (1949), Suter (1956), or Bloch (1961).

A. In Vivo Studies.

One of the earliest investigators to employ a satisfactory in vivo procedure for delineating the role of acquired cellular immunity in the absence of antibody was Lurie in 1942. Prior studies by this researcher had been published (Lurie, 1933 and 1939) dealing with cellular immunity against tuberculosis, but were inadequate in one or more ways. In the later study, however, he employed the anterior chamber of the normal rabbit eye as a culture chamber, eliminating many of the prior objections, such as the presence of immune fluids. The procedure involved obtaining

pleural exudates from normal and immune rabbits, then washing the cells to free them of any humoral components. These were next infected with tubercle bacilli in vitro in the presence of either fresh normal or immune serum. After ingestion the cells were washed free of extracellular organisms and the phagocytes containing ingested bacilli subsequently "planted" into the anterior chamber of the normal rabbit eye. After an appropriate period the number of organisms present in the eye was determined by plate counts. The results obtained by this method brought out two important points: (1) "immune" phagocytes inhibited the multiplication of the tubercle bacilli as compared to normal phagocytes, and (2) this occurred regardless of the presence of immune or normal serum. Therefore, it is here that was found the first clear-cut experimental evidence that phagocytes are capable of enhanced resistance, and that antibody appears to play at least a secondary role. It should be noted, however, that an immune humoral factor may play at least some role in tuberculosis immunity, as shown by Robson, Smith and Thomas (1960) and Fong, Schneider and Elberg (1956), and that host resistance is, as in all infectious diseases, very likely dependent on both cellular and humoral factors.

Although Lurie's study was criticized by Rich (1951) on the basis of unsatisfactory plate count methods as well as the fact that the human strain of tubercle bacilli employed was not pathogenic for rabbits, the results of Lurie (1942) do not stand alone in favoring cellular immunity. Indeed, Rich himself (1951) maintained the importance of mononuclear cells as the major protective mechanism against tuberculosis since other cellular and humoral factors are not known to destroy the causitive agent of tuberculosis.

Passive transfer of cells from immune to normal animals has more recently been employed to show the enhanced capabilities of "immune" phagocytes. Sever (1960) compared the mortality rates of mice infected with tuberculosis after passive transfer of normal or "immune" macrophages or spleen homogenates to these animals the day prior to, and 2 and 5 days after intraperitoneal challenge, with and without normal or immune plasma. The results indicated that the survival of animals receiving "immune" cells without immune plasma was significantly prolonged, and those receiving immune plasma as well as immune cells survived slightly longer than those receiving normal cells in the absence or presence of normal plasma. On the other hand, spleen cells or immune plasma alone failed to alter the mortality rates compared to untreated animals. Nakaguchi (1960), employing peritoneal macrophages from guinea pigs immunized with a heat-killed vaccine, and Millman (1962), employing phagocytes from normal and BCG vaccinated mice have obtained similar results. However, in the investigation by Millman, in vitro infection of the cells prior to transfer was found necessary, in contrast to the technique used by Sever (1960), since direct challenge of recipient animals did not lead to significant differences in mortality rates of the two groups. Likewise, Suter (1961) could find no differences in mortality rates of directly challenged mice that had received peritoneal cells from BCG vaccinated animals from those that had received normal cells. It is quite possible that in these three studies differences noted were due to varying strains of mice used as well as differences in strains of tubercle bacilli used for challenge. In the latter case, for example, Sever (1960) employed 1 mg wet weight of

the H37Rv strain of Mycobacterium tuberculosis for challenge; Millman (1962) used approximately 0.5 mg H37Rv per 2.9×10^6 mononuclear cells (infected in vitro); and Suter (1961) challenged recipients intravenously with 0.1 ml of a culture of the Vallée strain of the tubercle bacillus. These facts point to the importance of procedural differences in experimental studies.

Suter and Hulliger (1960) also passively transferred cells of lymph nodes, peritoneal cavity exudates, and spleen from M. tuberculosis (BCG and R1V1 strains) vaccinated guinea pigs to normal animals. The effect of this procedure was assessed by the extent of skin lesions produced through subsequent intradermal challenge with BCG and by the extent of pulmonary lesions produced by exposure to H37Rv strain of the tubercle bacillus. In all cases the extent of injury produced in the animals receiving cells from vaccinated animals was reduced when compared to those challenged after receiving normal cells. These results were recently confirmed by Suter (1961) through transfer of lymph node and spleen cells of BCG-vaccinated guinea pigs to normal animals, as well as by transfer of spleen or peritoneal exudate cells from BCG-vaccinated mice to normal mice. In addition, it was found that peritoneal exudate cells from recipients of phagocytes from previously vaccinated animals were more capable of inhibiting intracellular multiplication of the R1Rv strain of the organism in in vitro cultures than were those of normal cell recipients. This may indicate that these methods of assay for the effects of cellular transfer are more sensitive than using the rates of animal mortality.

Recently Fong and his associates (1962) used other means of assessing the effectiveness of transferred peritoneal histiocytes, lymph node cells and peritoneal polymorphonuclear leukocytes, as well as lysates of histiocytes and lymphocytes, from BCG immunized rabbits to normal animals. Prior studies by this group (Fong et al., 1956, 1957 and 1959) indicated that "immune" monocytes had the capacity to resist in vitro degeneration by the tubercle bacilli, but only in the presence of immune serum. Thus, in this latest study (Fong et al., 1962) washed cells from immune animals were transferred in the presence of immune serum to normal recipients by the intradermal route, then peritoneal macrophages from these latter animals were assayed in vitro in the presence of immune serum. The efficacy of the transferred cells was measured by the ability of the recipient cells to resist degeneration when infected with the H37Rv strain of the tubercle bacillus in vitro. By this method it was shown that "immune" histiocytes, lymphocytes, and histiocytic lysates, but not lymphocytic lysates nor intact polymorphonuclear leukocytes from donor animals were capable of reducing degeneration of the recipients macrophages. Intact histiocytes and lymphocytes from normal donors as well as normal or immune serum failed to impart this character to the cells of the normal recipient. Immune macrophages were better able to produce the observed effect than lymphocytes as shown by the requirement for fewer histiocytes to be transferred to reduce necrotization of the recipient's phagocytes. Furthermore, four "serial" transfers of cells (i.e. cells transferred from the immune animal to a normal recipient, then from this animal to another normal animal, and continuing at 13 day intervals until 4

successive transfers had been made) caused a loss of the ability to prevent necrotization from the second transfer (3rd recipient) following passage of "immune" lymph node cells, while "immune" histiocytes prevented degeneration through all transfers. This latter experiment, in addition, led to the hypothesis that the immunizing antigens were not being transferred with the initial passage of cells, since these would have been "diluted" out by the last transfer, thereby being incapable of producing the "cell resistance factor." This hypothesis was given some credence by the lack of stainable bacteria prior to transfer, and by the lack of production of a serum "protective factor" from recipients, which was known to be present on immunization with whole organisms. The role of serum in this study, as well as previous investigations by this group, would appear to indicate the participation of both cells and serum in tuberculosis immunity. However, Lurie (1960) has critized assay procedures such as those cited here since "... the obvious stricture should be borne in mind that, unless the in vitro conditions simulate the essential requisites of the former [in vivo conditions], tissue culture studies throw no significant light on the host-parasite relationships as they occur in the body." This statement alludes to the observation that normal animals may contain numerous tubercle bacilli in vivo without showing any injury, the latter phenomenon being the criteria of protection used by Fong et al. (1956).

Not all evidence has been in favor of cellular immunity in tuberculosis. A comprehensive study was carried out by Raffel (1955), who implanted semi-permeable capsules containing tubercle bacilli-infected

normal or "immune" monocytes into the peritoneal cavity of normal or vaccinated guinea pigs. Under these presumably optimal conditions, where the body fluids bathed the infected phagocytes, no differences could be detected in the intracellular multiplication rates of bacilli in the normal or "immune" cells. In addition, extensive experiments involving passive transfer of serum from vaccinated to normal animals did not alter the immunity of the recipients and serological data could not be correlated with resistance. It is apparent from these findings that the problem of elucidating cellular immunity in the case of tuberculosis by in vivo methods has not been entirely solved.

Other types of infectious as well as non-infectious agents have been employed to study Metchnikoff's theory in vivo. For example, McElree and Downs (1961) found enhanced cellular response to injection of killed Pasteurella tularensis after immunization with the homologous organism. Allen (1962) demonstrated that the passive transfer of peritoneal phagocytes, spleen cells, or lymph node cells from mice immunized with a living P. tularensis vaccine was capable of inducing increased resistance in recipient animals compared to those receiving cells from normal animals. This phenomenon was attributed to a property of the tissue cells and not to the presence of intracellular viable organisms, since the latter were shown to be absent by plate cultures. Furthermore, it was noted that immunization of the donors had to be carried out with viable homologous organisms since neither a killed P. tularensis vaccine nor a viable P. pestis vaccine sufficed to induce "cellular immunity" in donors in adequate amounts to provide increased resistance in the recipients on

subsequent challenge. It is also noteworthy that passive transfer of tissue from other organs of immune animals, such as lung, liver, and bone marrow failed to transfer resistance to the recipients.

Studies by von Litchenberg and Ritchie (1961) attributed the immunity developed by monkeys (Macaca mulatta) against Schistosoma mansoni, a blood fluke parasite, to both cellular and humoral factors. Conclusions concerning cellular participation were based largely on histological differences between normal and infected animals. No evidence distinguishing between capacities of cells from normal and infected animals is apparent in this work.

An outstanding study with regard to in vivo procedures was that of Donaldson et al. in 1956. Their technique involved the immunization of mice against chicken red cells and then exposing a portion of these animals to 350 roentgens (r) or 450 r x-irradiation to depress cellular activity but not affect antibody levels. Subsequently, the animals were challenged with an intraperitoneal injection of the red cells, then samples taken at periodic intervals and microscopically examined for the amount of cytopepsis of the cells by peritoneal phagocytes. It was found, as might be expected, that the phagocytes from non-irradiated immune animals exhibited enhanced cytopeptic activities over control non-irradiated animals. However, irradiation treatment of immune animals depressed cellular digestion even though antibody was maintained at a preirradiation level. It is interesting to note that the degree of cytopepsis observed was not significantly different from that of normal irradiated animals. Extension of these studies by Miya, Marcus and Perkins (1961) also gave

positive results employing bacterial agents. In this work peritoneal exudates were obtained from normal and Klebsiella pneumoniae or Salmonella typhimurium immunized mice and transferred to the peritoneal cavity of previously x-irradiated animals. The recipients were subsequently challenged with the organism being studied and the rates of mortality compared. In each case the group receiving the "immune" cells were protected to a significant degree compared to normal cell recipients. These results were found to correlate well with in vitro experiments carried out by the same group, which will be discussed below, and to give further supporting evidence of the importance of phagocytic cells in the disposition of foreign material and protection of the host.

Studies by Perkins and Marcus (1958) involving the role of preformed antibody through active immunization before x-irradiation or passively transferred antibody after x-ray treatment gives further insight into the importance of cellular mechanisms. It was determined in these investigations that active or passive immunity in mice resulted in similar mortality rates when the animals were treated with 0, 350 or 400 r x-irradiation followed by challenge with equal numbers of K. pneumoniae. However, at a critical point, 425 r, the mortality rates in the passively immunized group were significantly higher than in the actively immunized group while at 450 r both actively and passively treated animals were not significantly different from challenged controls. These results led to the hypothesis that antibody was effective only in the presence of functionally capable phagocytes, as might be the case in animals given less than 400 r x-irradiation. At 425 r the protection afforded by antibody was significantly

reduced as shown by the high mortality rates in the passively immunized group; however, since the mortality of actively immunized animals was significantly less the hypothesis was advanced that the remaining "immune" cells in this latter group were effectively depressing the infection. At the 450 r level of irradiation a critical number of phagocytic cells were apparently functionally impaired to the point where the infection led to similar mortality rates in all challenged groups regardless of the presence of antibody. Similar conclusions may be derived from the work by Nelson and Becker (1959 and 1960).

Further interesting points were brought out in passive cell transfer experiments described by Ushiba et al. (1959b). In this study glycogen-induced peritoneal cells were transferred from mice previously immunized with either attenuated living Salmonella enteritidis or a heat-killed vaccine prepared from a virulent strain of the organism to normal animals. These latter animals were subsequently challenged by the intraperitoneal route with the living virulent bacteria and the relative mortality rates observed and compared to that found in control animals. It was noted that for the phagocytes to afford protection to recipients they had to (1) be passed to the animal by the intravenous route, rather than by the subcutaneous route, (2) be obtained from animals immunized with the attenuated living vaccine, and (3) apparently contain viable intracellular bacteria at the time of transfer. Monocytes from the group of animals treated with the killed vaccine provided a small amount of protection when compared to untreated controls; that is, the degree of protection was below that provided by cells from animals treated with the living vaccine. The role

of intracellular bacteria in the transferred macrophages remains to be determined. However, it is of interest that among the macrophage cells obtained from animals immunized with the living vaccine, which were subsequently separated into those containing and not containing bacteria, only those that contained the organism when transferred to normal animals provided protection. Cells not containing intracellular bacteria failed to protect the recipients. Furthermore, it is recalled that Millman (1962) suggested that the presence of intracellular M. tuberculosis prior to transfer was important in imparting immunity to the host, a factor which also might have participated in Suter's (1961) experiments, since intracellular bacteria were noted in spleen and peritoneal cells transferred to normal animals. However, Fong et al. (1962), as previously mentioned, presented evidence that suggested the lack of importance of having antigens present in transferred cells, and Nakaguchi (1960) transferred resistance against tuberculosis to recipients by cells from animals immunized with a heat-killed vaccine.

The classical significance given to antibody in phagocytosis, particularly by the discoveries of Leishman (1902) and Wright and Douglas (1903), has been reaffirmed by modern work, such as that of Benacerraf, Sebastyen and Schossman (1959), who have been able to correlate blood clearance levels of injected P-32 labeled Escherichia coli and Staphylococcus aureus with antibody levels against these organisms. In addition, complement was found to be an important participant in this process (Benecerraf and Miescher, 1960). This is further discussed in Section III on the role of C' in phagocytosis.

Another approach to the study of acquired cellular immunity to poliomyelitis has been employed by Rebuck and LoGrippo (1961). They used human skin windows in immunized and non-immunized individuals such that a cellular response could be observed after application of the antigen to an area of insult. The characteristic response in the immunized individuals compared to normals was an earlier influx of lymphocytes and earlier transformation of these into macrophages. The authors suggested that this phenomenon in the immune population was probably due to "prior cytoimmunologic experiences in the lymphatic tissue..." giving increased leucocytic responses.

One of the few in vivo studies concerning cellular immunity against a mycotic agent has been that of Hill and Marcus (1960). In these experiments P-32 labeled yeast phase H. capsulatum were injected into normal and immunized mice. After 24 and 96 hours animals from each group were sacrificed, their spleens removed and assayed for radioactivity. The differences in amounts of emission of radioactive particles from the spleens of the two groups of animals were compared, giving an indication as to the relative numbers of organisms present. By this technique it was found that after 24 hours the radioactivity of the "immune" spleens was significantly less than that given by spleens from normal animals, but by 96 hours the tracer levels were approximately the same in spleens from both groups. The interpretation placed on this phenomenon was that cells in the "immune" spleens digested the phagocytized organisms more rapidly than did cells in normal spleens giving a lower count in the former after 24 hours. Continued cytopepsis by the normal spleens over

96 hours led to digestion of the contained organisms such that at the later time the levels of radioactivity of spleens of normal and immune animals were similar. It is noted, however, that in these particular experiments the role of antibody was not shown, although this factor was demonstrated through in vitro studies, to be discussed below, to perhaps play some role in mouse histoplasmosis.

It is apparent that studies carried out by in vivo methods suggest that acquired cellular immunity does exist. Furthermore such immunity is manifest with many types of infectious as well as noninfectious agents. It should be pointed out here that by the very nature of these studies the role of naturally occurring humoral factors such as complement (C') and properdin are assumed to be present, however, their effects on phagocytic activity remains to be determined.

B. In Vitro Studies.

1. Bacterial infections. In vitro methods for studying phagocytosis and intraphagocytic digestion have certain inherent disadvantages; for example, the always troublesome question of the relation to effects noted to that within the living host. Despite such objection, these procedures may be employed with many advantages. Among these advantages is the ability to separate the cells under study from the influence of host environment and to incorporate factors to be studied under well-controlled circumstances.

A large number of procedures have been described by various investigators, but as yet, none has become standard, making comparisons between various studies often times difficult. However, most methods do have

points in common. For example, peritoneal macrophages have been commonly employed. These cells, it is tacitly assumed, are washed free of surface humoral factors. The cells are then suspended in a type of tissue culture medium which usually contains some heated or unheated homologous or heterologous serum. Next, the phagocytic processes are studied, after infection of the cell with the agent under investigation, over a period of time by plate counts, microscopic observations, isotope procedures, or combinations of these.

The earliest in vitro study to show characteristic differences in normal and "immune" phagocytes was reported in 1953 by Suter using tuberculosis-infected rabbit and guinea pig peritoneal phagocytes from BCG vaccinated and normal animals. After suspending these in a menstruum of Hanks' balanced salt solution (BSS) containing 80% homologous unheated normal or immune serum, he was able to demonstrate by microscopic observations of samples taken at periodic intervals that the bacilli contained within the "immune" macrophages were inhibited in rates of multiplication compared to those in normal cells. The presence of immune serum did not appear to alter the final disposition of the tubercle bacilli when compared to that in normal serum. Further studies by this same investigator gave similar findings with cells from actively infected animals (Suter, 1955; Suter and Hulliger, 1960), as well as with peritoneal macrophages from rabbits injected with "purified wax" or lipopolysaccharides of the tubercle bacillus (Suter and White, 1954).

Confirmatory evidence for Suter's findings in tuberculosis have subsequently appeared. For example, Raffel (1955) found similar inhibition

of multiplication of tubercle bacilli by "immune" macrophages from guinea pigs, as did Abe (1958). Berthrong and Hamilton (1959) observed reduced intracellular serpentine cord formation of tubercle bacilli in "immune" cells compared to normal cells, which, incidentally, were cultured in guinea pig plasma clots using a menstruum of 40% normal guinea pig serum in Hanks' BSS. It is of interest that in the latter study inhibition of multiplication of the bacilli was not a satisfactory criteria for assessing the cellular effect. This might be explained by the differences in culture menstruums and procedures between this group and that of Suter (1953) or Raffel (1955).

It appears that phagocytes from animals vaccinated against tuberculosis may also exhibit other properties, although the significance of these acquired characteristics is not clear. A case in point is that of Stahelin et al. (1957), who found that the respiration rate of tubercle bacilli-infected liver slices was more rapid than that of infected slices from normal animals. However, under noninfected conditions the respiration rates of each tissue were equivalent. These results would serve to indicate that prior exposure of certain tissues to an antigen are more capable in a metabolic fashion. In addition, Patnode and Hudgins (1959) maintained that cells from guinea pigs that had previously been given heat killed or BCG tubercle bacilli were less susceptible to lysis by sonic vibrations than cells from normal animals. However, the importance of this phenomenon in understanding acquired immunity and the host-parasite relationship remains to be determined.

As in the case of in vivo studies, not all evidence favors the enhanced role of cells in immunity. Even though, as previously mentioned, Rich (1951) favored the role of cells he was unable to show any in vitro inhibition of the intracellular multiplication rate of tubercle bacilli by mononuclear spleen cells from vaccinated rabbits, guinea pigs, or fowl when compared to cells from normal animals. Also, Mackaness (1954), failed to distinguish between the multiplication rates of tubercle bacilli in infected monocytes obtained from normal and immunized rabbits. In comparing this study to that of Suter's (1953) it is noted that here the ingestion phase of infection was allowed to proceed in fresh rabbit serum. After infection in vitro, the cells were resuspended in Hanks' BSS containing 20% aged homologous serum (compared to 80% fresh serum employed by Suter). Furthermore, the degree of parasitization in this experiment was quite high (50-90% infected monocytes) compared with that employed by Suter (7-12%). Therefore, as pointed out by Suter (1954) it is probable that the variance in results was due to different techniques, although these differences do not appear to be large.

Employing carefully standardized procedures Fong et al. (1956) have studied the tubercle bacilli-monocyte relationship. Their procedure involved suspending washed peritoneal phagocytes from normal and BCG immunized rabbits in homologous serum. After parasitization with a low number of tubercle bacilli the infected cells are cultured in small tissue culture chambers in the presence of 40% aged immune or normal rabbit serum. Using phagocyte degeneration as an index of cellular incapacity it was observed that there were no significant differences between the two cell

populations in the presence of normal serum. However, immune serum was found to inhibit cellular degeneration in normal and "immune" phagocytes, but more so in the latter. This would appear to indicate that this serum contained an important factor not heretofore described in the case of tuberculosis. As a matter of interest, subsequent study by this group has shown that this factor is nonspecific in action (Fong et al., 1957) and not characteristic of antibody, complement or properdin (Fong et al., 1959; Elberg, 1960).

Brucella infections have also been subjected to in vitro studies in cellular infections with the appearance of positive differences. Pomales-Lebron and Stinebring (1957) for example, demonstrated by plate counts that peritoneal cells from immunized guinea pigs suspended in Hanks' BSS and 30% normal guinea pig serum were able to suppress the intracellular multiplication of Brucella abortus, while normal cells permitted an increase in bacilli. This finding was confirmed by Holland and Pickett (1958) in mouse, rat and guinea pig peritoneal cells in the presence of either immune or normal serum.

In studies of Pseudomonas infections, Nelson and Becker (1959 and 1960) were able to show that, under certain circumstances, "immune" cells from mice killed the bacteria more rapidly than normal cells even in the absence of serum factors. The predisposing condition was the exposure of the animals to at least 300 r x-irradiation prior to infecting the cells. This may be interpreted to mean that, in the case of this organism, the lethal activity of phagocytes is quite potent in both normal and immune mice and distinguishing differences in the cell populations

do not appear unless the animals are under stress or cannot be observed under the experimental conditions unless certain participating cellular factors are suppressed as appeared to be the case in other work of similar design (Perkins and Marcus, 1958). This may have been one explanation why Morello and Baker (1961) failed to find little or no difference between the cellular activities of normal and "immune" phagocytes from mice infected with Salmonella typhosa.

Gelzer and Suter (1959) were unable to distinguish between the relative actions of rabbit mononuclear cells from normal and immunized animals on S. typhimurium when the phagocytes were infected in vitro, provided the cells were well washed to remove any humoral factors. In the presence of antibody, however, intracellular multiplication of the organisms was retarded compared to that in the absence of antibody. In this case, then, humoral and cellular factors were apparently interdependent and both were necessary before bacteriostatic characteristics of either were apparent.

On the other hand, Saito et al. (1960) showed that peritoneal phagocytes from mice and guinea pigs vaccinated with an attenuated living vaccine produced significant suppression of intracellular multiplication of S. enteritidis in vitro without the presence of immune serum when compared to proliferation of the organism in phagocytes from either normal animals or those vaccinated with a heat-killed virulent strain of the culture. The macrophages from animals given the heat-killed vaccine did, however, produce slight inhibition of multiplication. This was most pronounced in the case of mouse cells when immune serum was present, although antibody was not found to alter results under any other circumstances when

compared to its absence. Similar findings were subsequently reported by Mitsuhashi, Sato and Tanaka (1961), and may indicate the necessity of employing a live vaccine in mice and guinea pigs to produce a high level of immunity, as suggested by Ushiba et al. (1959 a). However, Gelzer and Suter (1959) employed a combination of killed and living organisms to immunize rabbits without clearly demonstrating cellular immunity.

Another experimental approach was employed by Miya et al. (1961) to study cellular resistance against K. pneumoniae and S. typhimurium. Here, rabbits were immunized with the organisms being studied, then peritoneal phagocytes were obtained and suspended in Warburg flasks. After infection of the cells with organisms, oxygen uptake was measured. The hypothesis examined was that the more capable the phagocytes, the greater the inhibition of oxygen uptake of the bacteria by the cells. This appeared to be upheld in these investigations, since immune infected cells did suppress O_2 uptake in all cases when compared to normal infected cells. Also, results correlated well with plate counts carried out on cell cultures after termination of the Warburg experiments; that is, fewer viable bacteria were recovered from "immune" cells than from normal cells.

Intraphagocytic infections with Listeria monocytogenes have been subjected to analysis by Osebold and Njoku-obi (1961) using sheep phagocytes. In their system suppression of multiplication of the organism took place in "immune" cells only in the presence of antibody while "immune" cells in normal serum yielded marked increases in bacterial numbers. Therefore, the role of cellular activity may be minimal in this infection.

2. Mycotic infections. Infections due to certain mycotic agents,

such as blastomycosis and histoplasmosis, may be chronic in nature and bear similarities to tuberculosis. As previously summarized, investigations into the latter disease give a basis for the hypothesis, though not unanimously, that acquired cellular immunity plays a predominant role in subsequent protection of the host. This might well be expected in the tuberculosis-like mycotic infections, and certain investigators appear to verify this through in vitro studies. Hill and Marcus (1960) for example, were able to quantitatively show a positive difference in the cytopeptic rates by phagocytes from normal and immune mice employing P-32 labeled H. capsulatum, but not in rates of phagocytosis. The procedure involved here was the maintenance of phagocytes attached to glass during which they were allowed to phagocytize the labeled organisms in the yeast phase. Phagocytic (ingestive) activity was followed by determining the rate of decrease of radioactivity in the medium and the cytopeptic phase subsequently expressed itself as an increase in the label in the medium. This process, it was hypothesized, was due to uptake of the labeled organisms by the attached phagocytes giving decreasing levels of radioactivity in the medium; the subsequent increase in supernatant activity in the medium was due to intracellular release of the labeled phosphorous from the yeast-phase organisms due to phagocytic digestion. The hypothesis appeared well-founded since plate counts and microscopic observations correlated with those found by the radioisotope procedure. Incidentally, it should be noted that the cell maintenance fluid employed in this study consisted of 20% heated (56° C) calf serum in a 10% lactalbumin hydrolysate, 70% Earle's BSS solution (Hill and Marcus, 1960). Here then, positive results

were obtained in a completely heterologous system (mouse phagocytes maintained in calf serum solution) in the absence of heat labile factors and antibody.

Extension of these studies were later carried out by Miya and Marcus (1961) employing slight modifications of the procedure described by Hill and Marcus (1960). These changes involved varying rabbit or guinea pig serum concentrations in the menstruums, depending on the conditions required, and deleting lactalbumin hydrolysate. Under the experimental circumstances enhancement of digestion of H. capsulatum was shown by immune mouse cells over normal cells only in the presence of a heat labile factor, such as complement or properdin. Therefore, in this regard, results differ from those obtained by Hill and Marcus (1960) where dissimilarity was observed in the absence of heat labile factors. However, confirmation of the comparable phagocytic rates of normal and "immune" cells obtained by the latter group was made by Miya and Marcus (1961). In addition, it was noted that specific rabbit antibody did not alter the results as compared to those obtained when normal serum was used.

The major difference in results in these two studies is that in one evidence was obtained that cellular immunity can be expressed without the presence of heat labile factors of calf serum, while in the other differences in normal and immune cell populations appeared only in the presence of heat labile factors of guinea pig or rabbit serum. The explanation for this is not clear, but it may indicate the significance of varying an established technical procedure.

3. Viral infections. Several studies have been carried out to

determine the role acquired cellular immunity might have on viral resistance. For example, Boand, Kempf and Hanson (1957) found influenza virus to be phagocytized by immune cells from humans and rabbits in the presence of normal serum, while normal cells in the same serum failed to take up the virus. Ginder (1955) also described enhanced neutralization of fibroma virus in the presence of peritoneal, lymph node, liver or spleen cells from immunized rabbits in the presence of unheated immune serum compared to normal cells in the same serum. Furthermore, in the case of peritoneal phagocytes "immune" cell lysates failed to give equivalent neutralization indices indicating the need for living phagocytes.

4. Protozoan infections. In vitro studies dealing with cellular immunity to protozoan parasites are few in number. One of note was reported in 1954 by Vischer and Suter using Toxoplasma gondii. Under the conditions employed "immune" cells from guinea pigs, rabbits, and rats in normal serum were able to inhibit intracellular development of the parasite to approximately the same degree as normal cells in immune serum. This correlated well with the in vivo study of S. mansoni reported by von Lichtenberg and Ritchie (1961) mentioned earlier, and may imply that both cellular and humoral factors are of equal importance in these types of infections.

Most of the above studies, both in vivo and in vitro, appear to be generally favorable toward acquired cellular immunity. The phenomenon is apparently applicable in many types of infections, both chronic and acute, but seems to vary in prominence between infections. Cellular immunity is apparently quite active, for example, in chronic bacterial and mycotic

infections where antibody apparently plays a minor role, while in the protozoan infections studied it participates at a maximal level only in the presence of antibody. In acute bacterial infections cellular immunity has been shown to be active, while in viral diseases its role is uncertain in the absence of antibody. This review does show in addition that in vivo and in vitro studies correlate well in overall results. However, it is apparent that in both cases, procedural details are important.

III. The Role of Complement in Phagocytosis and Digestion

Complement (C') is a naturally-occurring heat labile (56° C for 30 minutes) serum factor which has been found through immune hemolysis studies to consist of at least five separate components. These are usually designated C'1, C'2, C'3a, C'3b and C'4, and require the presence of Mg^{++} and Ca^{++} to exert their hemolytic action (Kabat and Mayer, 1961).

A large amount of information exists concerning the function of C' in serologic reactions, however little is known concerning its in vivo action or chemical nature (Kabat and Mayer, 1961; Osler, 1961). With regard to serologic activity for example, it is known that this heat labile factor participates in the in vitro immune hemolysis of antibody-sensitized erythrocytes and in killing certain sensitized bacteria and protozoa (Kabat and Mayer, 1961); C' also takes part in the immune-adherence phenomenon (Nelson, 1953) and may play a role in certain autoimmune diseases (Robineaux and Pinet, 1960; Lachman et al., 1962). Furthermore, it may be involved in phagocytosis (Ecker, 1948; Skarnes and Watson, 1957) and possibly the cytopepsis of certain microorganisms (Miya and Marcus, 1961). In fact, according to Heidelberger (1951), complement plays its most important role

in promoting phagocytosis in infections by combining with the infectious agent and antibody. In this section of the literature review I shall attempt to elucidate the role of complement in ingestion and digestion of particulate matter by phagocytes, insofar as is known at this time.

A. The Participation of Opsonins in Phagocytosis.

Before proceeding with the subject of the participation of complement in phagocytosis and cytopepsis it appears necessary to review some of the evidence pertaining to the function of the heat stable factor involved in this process, since this as well as C' appear to be intimately involved in phagocytosis. It should be noted that this portion of the literature review is not a complete survey, but only sufficient to give the proper perspective for the involvement of complement which is to be summarized in the next sections.

Among the earliest investigators to implicate heat labile humoral factors in enhancing phagocytosis were Wright and Douglas in 1903, employing a method modified from that originally described by Leishman (1902). The procedure involved mixing phagocytes, bacteria and serum or plasma together, incubating at 37°C for definite periods, and then preparing stained smears of these mixtures. The number of bacteria contained within a certain number of phagocytes were then enumerated by microscopic observations. The average number of bacteria per phagocyte was computed and called the "phagocytic power" or more commonly known today as the "phagocytic index." Comparisons between the effects of various sera could be made on this basis, although the "opsonic index," which is obtained by dividing the phagocytic index values of a serum sample in

question by the phagocytic index of normal serum, can also be used (Carpenter, 1956).

Using this procedure Wright and Douglas (1903) found that staphylococci were not taken up to any great extent by human leukocytes in the presence of heated normal serum, (i.e. a low phagocytic power) as compared to that obtained in the presence of unheated normal serum. Furthermore, the activity exerted by unheated serum was decreased upon standing at room temperature. It appeared to these workers that they had discovered a new type of antibody which was heat labile and coined the term "opsonin" to describe it.

Further investigations described in a series of papers by Dean (1905, 1907a and b) appear to implicate heat stable factors as well as heat labile factors in aiding phagocytosis. For example, Dean noted that heating normal horse serum decreased the phagocytic index using staphylococci and other organisms, but did not completely abolish opsonic activity (Dean, 1905). Later, it was noted that fresh rabbit serum enhanced the uptake of staphylococci or the dysentery bacillus when added to heated anti-staphylococcal or anti-dysentery bacillus serum, respectively. Furthermore, when heated normal rabbit serum was mixed with unheated normal guinea pig serum and typhoid bacilli the phagocytic index was increased to values higher than obtained with either of the two sera used alone. Anti-complement, prepared by injecting rabbits with fresh horse serum, also caused decreased uptake of staphylococci with fresh horse serum and human phagocytes as compared to that given when the anti-C' was not used in the mixture. A combination of staphylococci, anti-C',

fresh human serum, anti-staphylococci and rabbit phagocytes gave a similar decrease in uptake of the organism as compared to when anti-C' was deleted (Dean, 1907a).

These experiments led Dean (1907b) to believe that there were actually two factors involved in phagocytosis. The first was heat stable and identical to immune antibody and the second was heat labile and probably C'. The level of opsonin, the term which this investigator applied to the heat stable factor, was low in normal unheated serum, but its action was enhanced by C'.

Conversely, Levaditi and Inmann, in 1907 (Zinsser, Enders and Fothergill, 1940) presented evidence that opsonin and C' were similar in that both were absent from the anterior chamber of the uninjured eye, while both enter the aqueous humor upon injury. Muir and Martin (1906) also came to similar conclusions when it was found that absorption of serum with antigen-antibody complexes removed hemolytic C' as well as opsonins against staphylococci. Other lines of investigation have led to similar conclusions concerning the similarity of C' and opsonin (Neufeld and Hune, 1907; Ruediger and Davis, 1907).

Confusion thus arose as to whether the factor involved in opsonization was actually heat labile as hypothesized by Wright and Douglas (1903) or was a combination of heat stable and heat labile factors as suggested by Dean (1907b) and others (e.g. Neufeld and Rimpou, 1904; Cowie and Chapin, 1907a and b, and Chapin and Cowie, 1907).

The problem of distinguishing the presence of the heat stable factor and to show its role in opsonization has been approached in several ways.

Studies involving measurement of opsonic activity of heated normal or immune sera after absorption with specific antigens, for example, served to demonstrate both heat stability as well as specificity of opsonins. Other methods have been used which attempted to show the similarity of opsonin in normal serum to agglutinating, precipitating, or complement fixing immune antibody.

The specificity of the heat stable factor, for example, was shown in experiments by Bulloch and Western (1905-06). These workers found that when normal human serum was absorbed with S. aureus or Escherichia coli, the respective opsonins for each of these organisms were specifically removed without altering the opsonic level to the other organism. The same specificity held when the tubercle bacillus was used for absorption leaving the opsonin against S. aureus. In addition, tuberculin injections into human patients caused a specific rise in opsonins for the tubercle bacilli without altering the opsonizing level for staphylococci.

Specificity of opsonins was also acknowledged by Wright and Reid (1905-06), since absorption of serum from tuberculosis patients with the tubercle bacilli removed the opsonin which enhanced uptake of the organism by phagocytic cells. In addition, by comparing the relative opsonic actions of immune and normal sera after heating and dilution, these workers concluded that the factor enhancing phagocytosis was the same in each sera. Other investigators have shown similar specificity of opsonins in vitro using staphylococci (Chapin and Cowie, 1907), pneumococci (Sia, 1926-27), and red blood cells (Mabry et al., 1956). However, the work of Ward and Enders (1933) is most instructive in this regard. These

workers were able to demonstrate that absorption of normal human serum with a specific carbohydrate antigen common to only one type of pneumococcus caused inhibition in uptake of only the organism containing this antigen. Also, in absorbing the serum with one type of pneumococcus the opsonin against a closely related type was not removed.

Conversely, the characteristic of non-specificity of reaction of opsonin has been demonstrated by Muir and Martin (1906). They employed sensitized ox erythrocytes to remove phagocytosis enhancing properties from normal guinea pig serum. This absorbed serum was examined for hemolytic as well as opsonic properties, detection of the latter being made with an antigen (staphylococci) unrelated to that used in the absorption of the serum. Their results indicated that the staphylococcal opsonin was removed by the preliminary absorption with heterologous antigen giving evidence that opsonins were apparently nonspecific. In addition to this, Simon, Lamar and Bispham (1906) found that absorption of normal serum with S. citreus failed to remove either the opsonin against S. citreus or that against the colon bacillus, leading them to believe that the opsonic factor was nonspecific.

Despite these latter studies, the specificity of the heat stable factor appears to be well established, both from the quantitative evidence presented by Ward and Enders (1933), and from results of others to be described later which show the similarity of this factor to immune antibody which is also specific in nature.

The heat stability of the opsonin in normal serum has been difficult to distinguish because of its low levels in such serum and because of the

phenomenon of "spontaneous phagocytosis." The latter is a process of phagocytosis of particles in the absence of any opsonin, such as that in the presence of physiological salt solution. Indeed, the change in the concentration of salt may in itself alter the amount of spontaneous phagocytosis (reviewed by Mudd, McCutcheon and Lucké, 1934).

Regardless of these difficulties Dean (1905), as mentioned previously, was able to show the heat stability of opsonins in both normal and immune serum. In addition, Chapin and Cowie (1907) presented evidence that S. albus, when treated with heated (56°C for 10 minutes) normal serum, was taken up slightly faster in the presence of heated serum than the untreated organism. Addition of diluted unheated serum caused marked increases in phagocytosis of the sensitized cocci, but had little effect on untreated organisms leading these workers to believe that a heat stable factor was present. Ward and Enders (1933) also came to similar conclusions from their studies when they found that both normal and immune sera contained heat stable opsonins against types I, II and III pneumococcus.

Other investigations have also indicated that the heat stable factor involved in opsonization is probably the same as antibody which manifest other properties such as agglutination, precipitation or hemolysis. This was shown, for example, by Delves (1937), who employed a rabbit anti-human albumin and human albumin system to test for agglutinating (by absorbing the protein antigen onto collodion particles), precipitating, complement fixating and opsonizing antibodies. It was noted that absorption of the antiserum with the protein, thereby giving a precipitin reaction, reduced or removed the antibody for the other reactions. Likewise,

Castelli (1942) has noted that the phagocytic index of the serum of animals being immunized rises and falls parallel to the agglutinating titer.

More recently, Benacerraf, Sebastyen and Schossman (1959), in quantitative in vivo studies of blood clearance of P-32 labeled Escherichia coli and S. aureus, showed that there was a definite relationship between the rates of disappearance of these organisms from the blood circulation after injection of the organisms and the agglutinating titers of normal and passively immunized animals. For example, it was found that the clearance of staphylococci was more rapid in normal mice than that of E. coli, and this finding was correlated with a higher natural antibody titer against the former organism. Also, in passive antibody transfer experiments in mice there was a direct relation between the amount of antibody injected and the uptake of E. coli up to a maximum rate, after which further increases in antibody against the organism did not lead to further increases in uptake. Biozzi et al. (1961) obtained comparable findings with I-131 labeled Salmonella typhosa. These results are similar to those of experiments carried out by Manwaring and Fritschen (1923), who perfused dog livers with Ringer's solution mixed with Bacillus anthracis or B. lactis aerogenes (Aerobacter aerogenes) and various sera. Phagocytic rates were measured by the difference in the number of viable bacteria in the organ before and after perfusion. Their findings showed that immunization of the animals before perfusion increased the amount of uptake by the liver as did adding immune serum to the perfusion fluids passed through normal livers. In addition, presensitization of B. lactis aerogenes (A. aerogenes) suspended in Ringer's solution gave increased

uptake by normal livers compared to nonsensitized organisms in the same medium. It is interesting to note also that when these sensitized organisms were passed through a normal spleen in the same fashion increased opsonization also took place, but not when passed through a normal lung.

Thus, it appears from the evidence presented here that heat stable antibodies are apparently involved in opsonization. With this point established, the role of C' will now be reviewed.

B. The Participation of Complement in Phagocytosis.

The early evidence that heat labile factors participated in phagocytosis consisted largely of observations that unheated normal serum could activate the opsonins of heated serum. This was demonstrated by Dean (1905), as previously mentioned, where staphylococci, typhoid, and dysentery bacilli were taken up at marked rates by phagocytes in heated normal serum after the addition of unheated normal serum. Heated serum alone was only slightly active in this respect. Hektoen (1908) also found that rat erythrocytes suspended in heated dog serum were taken up by dog mononuclear phagocytes to a greater extent when unheated serum was added to the mixture. In a later study this same investigator showed that the uptake of anthrax bacillus by dog phagocytes was actually dependent on the presence of unheated serum since heated serum alone would not opsonize the bacteria (Hektoen, 1909). Similar activation of opsonins by unheated serum has been reported by Hektoen and Ruediger (1905), Cowie and Chapin (1907a and b) and Eggers (1908). Conversely, Sellards (1908) was not able to show enhanced opsonizing effects on organisms suspended in unheated normal serum as compared to organisms pretreated with this

serum followed by heating at 60°C. However, as pointed out by Zinsser et al. (1940) the presence of complement in the unheated serum may have led to a rapid union of the antibody with the antigen such that rapid phagocytosis subsequently took place regardless of heating of the sensitized organism.

Lucké et al. (1933) compared rates of phagocytosis of an avirulent strain of M. tuberculosis suspended in the presence of immune or normal rabbit serum. Their findings indicated that heating immune serum did not decrease the rates of uptake of the organism, while the same treatment of normal serum caused ingestion to be markedly decreased as compared to unheated normal serum. The opsonic effect of the normal serum, however, was diluted out much sooner than that in immune sera. Similarly, results obtained by Howard and Wardlaw (1958) through perfusion studies on rat livers, showed that heating immune serum did not cause decreased uptake of E. coli or K. pneumoniae by phagocytes. Heating of normal human, mouse or rat serum, however led to marked decreases in uptake, although the amount of phagocytosis noted under these conditions was still higher than with Ringer-Locke solution alone.

In the studies of Chapin and Cowie (1907) absorbed unheated normal serum was found to retain the capacity to activate either heated serum or organisms that had been treated with heated serum. However the absorbed unheated normal serum was not opsonic by itself, nor could it activate absorbed heated serum. Furthermore, Howard and Wardlaw (1958) showed that absorbtion of the serum with bacteria which did not decrease C' levels caused reduced uptake compared to unheated and unabsorbed serum,

but levels were still above that of heated serum. Heating and absorption of normal serum reduced uptake to the level given by salt solution alone. The slight uptake of organisms obtained with unheated absorbed serum was not attributed to C', but rather to another heat labile factor, properdin. This was shown by treating serum that had previously been absorbed with bacteria with zymosan at 17°C. This procedure selectively removes properdin without removing all the C' (more than 50% of the C' remained after treatment in these studies). Subsequent mixture of this serum with bacteria showed that the serum was now incapable of opsonizing over levels obtained with salt solution alone.

The importance of complement in opsonization was further demonstrated by Moore (1919), using guinea pigs naturally deficient in this factor. Employing serum from these animals it was found that the opsonic index and phagocytic index were subnormal compared to normal guinea pigs. The impact of this deficiency on the natural immunity of the animal was noted by the observation that the C' deficient animals were more susceptible to challenge with Salmonella cholerae suis. Subsequent studies by Coca (1920-21) on a C' deficient colony of animals indicated that it was actually the limiting amounts of the third component of complement (C'3) which accounted for this deficiency of C' in these animals.

Artificial means have subsequently been employed in attempts to decrease C' levels in vivo and in vitro. For example, Jungeblut and Berlot (1926) found that injections of India ink into guinea pigs caused C' levels to drop within 15 minutes, reaching a low after 3 hours, after which the titer slowly rose back to the original level within 24 hours.

Drawing upon the results of this experiment, Elvidge (1933) injected quartz particles into rabbits and noted that opsonic action of serum subsequently obtained from these animals decreased after 7 to 12 hours and lasted for several days. Thus, the opsonic level found by this investigator and altered complement level as noted by Jungeblut and Berlot (1926) did not appear to coincide. However, it is difficult to rationalize the experiment of Elvidge's (1933) since he attempted to integrate the results of Jungeblut and Berlot into his experiments, even though the species of animals used, as well as the particles used to inhibit C' were different.

Biozzi and Bier (1961) attempted to "decomplement" mice and rats by injecting antigen-antibody complexes. They were partially successful with rats in that only 10% of the original C' activity was measurable one hour following administration and about 30% was measurable for the following three hours. The effect of the lack of C' on blood clearance of injected carbon particles was then studied and compared to results obtained from normal animals. No significant differences could be found between the two groups of animals. It is possible that these investigators actually "decomplemented" their normal animals by the carbon particle injections (Jungeblut and Berlot, 1926) thereby eliminating the supposed differences in C' levels between the "normal" and treated groups of animals.

The participation of complement in in vivo clearance of *E. coli* has been further explored by Benacerraf and Miescher (1960) in mice. They noted that these bacteria in combination with specific rabbit antibody were cleared from the circulatory system very rapidly, although a short lag (4 minutes) in uptake was noted before the sharp increase in rates

was observed. With chicken antibody, however, it was found that a much longer lag period occurred (15 minutes) before rapid clearance proceeded. To further analyze this phenomenon the organisms were incubated at 37°C for 30 minutes with chicken antibody and either normal unheated mouse serum, heated mouse serum, or mouse serum treated with zymosan at 37°C which selectively binds C'3. Subsequent injection of the treated organisms into mice showed that preincubation of the sensitized cells in untreated normal mouse serum led to immediate clearance of the organism. Those pretreated with mouse serum where C' had been inactivated by heating or zymosan treatment, on the other hand, resulted in 15 minute lag periods in uptake previously observed with E. coli sensitized with chicken antibody injected without benefit of in vitro preincubation. Thus, the lag period was attributed to the time necessary for the mouse C' system to complement the opsonizing antibody in vivo. Biozzi et al. (1961) recently observed a similar lag period in in vivo clearance of labeled S. typhosa in mice passively treated with rabbit antiserum and attributed the phenomenon to the mechanism hypothesized by Benacerraf and Miescher (1960).

Conversely, evidence has been presented that whole C' is unnecessary for enhancement of opsonization. This was concluded from the fact that treatment of C' with ammonia, specifically removing C'4, (Gordon, Whitehead and Wormall, 1926a), inactivated the hemolytic property of C' in immune hemolysis without altering opsonizing properties (Gordon, Whitehead and Wormall, 1926b and 1929; Gordon and Thompson, 1935). However, this does not appear to exclude the possibility that C'1, 2 and 3 may bring about enhancement of phagocytosis, or that C'4 may actually be supplied by

leukocytes as shown by Maltaner (1935) and Gordon (1937). However, the action of congo red, which also inactivates hemolytic C' without inactivating opsonins (Gordon, 1930; Gordon and Thompson, 1935) remains to be explained, although the action of the dye with regard to opsonins may be questioned since Castelli (1942) found that both C' and opsonin were inactivated by this substance.

Several in vitro studies have been carried out which implicate complement further in phagocytosis. A much cited investigation in regards to the role of C' has been that of Ward and Enders (1933), who did much to advance the knowledge concerning the participation of C' as well as to show definitively the specificity of the heat stable factor, as previously discussed. In elucidating the function of the heat labile factor these investigators found that normal human serum could opsonize pneumococci, but that this activity was lost on heating. However, by mixing the nonopsonizing heated serum with infants serum, the latter containing no opsonins by itself while having high levels of C', the opsonizing capacity of the heated serum was reactivated. Complement alone would not opsonize the organism as found by mixing the bacteria with infants serum. Furthermore, on investigating the effects of C' on rates of ingestion of pneumococci sensitized with rabbit antiserum it was found that the heat labile factor increased the number of organisms taken up with time, but did not increase the total number of bacteria taken up when compared to the action of antibody alone. These latter findings have since been confirmed by Miya and Marcus (1961) employing P-32 labeled H. capsulatum, and by Jeter, McKee and Mason (1961) with pneumococci and rabbit anti-complement.

However, Welch, Brewer and Hunter (1940) could not show increased rates of uptake with sensitized staphylococci in the presence of C' although it was noted that C' alone could not opsonize these organisms. This was shown by treating normal plasma with certain antiseptics, such as I_2 and $HgCl_2$, which acted upon the opsonin at certain concentrations without inhibiting the phagocytes or causing a loss of hemolytic C'. Plasma thus treated would not opsonize, even though hemolytic C' was present in high concentrations (distinguishable in 1:1000 dilution) and addition of this treated C'-containing reagent to heated guinea pig serum and sensitized staphylococci did not lead to phagocytosis.

In contrast, a study by Maaløe (1947) has shown the importance of C' in uptake of Salmonella breslau. As little as a 1/1000 dilution of serum gave enhanced uptake of this organism in the presence of antibody, even though a 1/10 dilution of C' gave no enhancement of uptake without antibody. Bonnin and Schwartz (1954) showed comparable results with sensitized erythrocytes and C' and Mabry et al. (1956) found some correlation in the amounts of C' present and the phagocytic activity of rabbit macrophages ingesting erythrocytes. Similarly, Lange and Lysenko (1960) found that phagocytosis of Trypanosoma lewisi was reduced after heating immune serum at 46°C for 30 minutes.

In interesting studies by Robineaux and Pinet (1960) the role of complement in lupus erythematosis, a disease believed by many workers in the field to be due to autosensitization, was subjected to analysis. Phagocytosis is involved in this disease since polymorphonuclear leukocytes take up masses of other nuclei, usually from disrupted lymphocytes and

neutrophils; the lysis is believed to be due to a serum factor identical to antibody. The resulting leukocytes which take on special configurations such as rosettes, are commonly called L. E. cells. C' is an integral portion of the antigen (nuclei of disrupted cells)-antibody complex and necessary for phagocytosis. Robineaux and Pinet (1960) actually noted that any cytoplasm attached to the nucleus of the disrupted cells was "disattached" by the PMN's in the ingestion process, apparently because unlike the nuclei this portion was not opsonized.

Nelson (1956) has also involved C' in phagocytosis in the "immune-adherence" or "I-A" reaction. This phenomenon is characterized by the participation of antibody and C' in causing bacteria or virus to become attached to the surface of normal red cells of certain mammals (Nelson, 1953). When this has occurred, it was found that phagocytes could "strip" the red cell of its adhering particles without damage to the erythrocytes (Nelson, 1953, 1956). Actually, the role that C' plays here appears indirect as compared to phagocytic reactions previously discussed. In fact, the mechanism seems to be similar to immune hemolytic reactions in requiring the participation of all components of complement (Nelson, 1956), a finding which may not necessarily be true in direct phagocytosis, as will be discussed below.

Despite the evidence favoring the participation of complement in phagocytosis, some investigations have shown its lack of importance. Gelzer and Suter (1959), for example, showed that C' and antibody, used separately or together, had little effect on the per cent of rabbit mono-nuclear cells ingesting S. typhimurium or on the final number of organisms

taken up by these cells. Also, Tullis and Surgenor (1956), studying phagocytosis of starch granules, characterized a heat labile factor that was found in both serum and plasma. Although this factor had certain characteristics common to C' and properdin, zymosan absorption at 17°C to remove properdin and subsequent treatment to inactivate C' did not reduce the activity. Therefore, it was concluded that the substance(s) was not either of these factors and was given the name "phagocytosis-promoting factor" or "PPF".

Regardless of the latter papers, it is apparent that the evidence favoring the involvement of complement in ingestion of microorganisms both in vivo and in vitro is substantial. Varying results presented by certain investigators are to be expected because of the complexities of the systems involved.

C. Complement Components Involved in Phagocytosis.

The fact that complement consists of more than one fraction was realized in 1907 when Ferrata dialyzed guinea pig serum against distilled water and found that the C' activity was contained in a soluble "pseudoglobulin" (endpiece, containing primarily C'2 with some C'3 and C'4) and insoluble "euglobulin" (midpiece, containing primarily C'1 with some C'3 and C'4) (Kabat and Mayer, 1961). This information led to an early investigation into the relative importance of midpiece (M) and endpiece (E) in phagocytosis, and is continuing at present. However, the problem now has been extended to include the relative roles of C'1, 2, 3 and 4, these components being identified as making up C'.

Possibly the earliest study in these regards was that of Simon et al. (1906). These workers dialyzed human serum against water which resulted in loss of opsonic activity. They noted that by making the dialyzed material isotonic they could restore opsonic activity of the euglobulin but not of the pseudoglobulin portion. From this it could possibly be implied that C' activity exists in the euglobulin; however, the partition of this from heat stable factors is insufficient since the latter is also found in the euglobulin fraction.

Zinsser and Cary (1914) fractionated guinea pig serum into M and E, then tested each fraction for opsonic power using S. aureus and guinea pig leukocytes. They found that the opsonic power resided in endpiece (C'2, 3 and 4), although it was noted that recombination of E and M did not lead to opsonization, even though the hemolytic power was restored. Ledingham and Dean (1911-12) observed similar complementary action of endpiece when added to a suspension of S. typhosa and human phagocytes.

Attempting to quantitate more thoroughly the role of C' and C' components Ecker, Weisberger and Pillemer (1942) carried through a series of studies where variables such as salt and serum concentration, temperature of incubation, and spontaneous phagocytosis were carefully controlled. When experiments were carried out using M and E of various sera, it was found that the thermolabile factor resided in either one or both fractions depending on the species from which the serum was obtained. Similarly, zymin treatment to remove C'3 also caused certain sera to lose opsonic activity without affecting others (Ecker, Pillemer and Kuehn, 1942). With human serum, M and E were both found to be opsonically inactive as

was R4 (complement deficient in C'4) while R3 (complement deficient in C'3) was active in this respect (Ecker and Lopez-Castro, 1947). Mabry et al. (1956) also regarded C'4 important in the ingestion of virus-treated erythrocytes and found some correlation in the level of C'4 and the phagocytic index. R3 on the other hand gave less inhibition of opsonization, indicating that C'3 is not essential.

D. The Role of Complement on the Intracellular Fate of the Microorganisms.

Few studies have been carried out concerning the effect of C' on intracellular fate of microorganisms. The results of these studies are conflicting.

Gelzer and Suter (1959), using rabbit mononuclear phagocytes, studied the effects of antibody and complement (fresh rabbit serum) on the inhibition of intracellular multiplication of S. typhimurium. They found that while antibody had the capacity to inhibit growth of the organism within the phagocyte, the presence of C' was unable to alter the effect.

In a later study, however, Miya and Marcus (1961) implicated heat labile factors in cytopepsis of P-32 labeled H. capsulatum in phagocytes of normal and immunized mice. Here it was found that fresh rabbit serum caused enhancement of digestion of the organism in both populations of phagocytes over that obtained with heated serum, but was highest in cells from the immune animals. It should be pointed out, however, that properdin could have been a participant in the enhancing process along with C', but results regarding this point were equivocal.

IV. The Role of the Properdin System in Cellular Resistance.

Before entering into a review of the role of the properdin system in phagocytosis and digestion it appears necessary to give a short summary of the characteristics of this system. This is presented only for orientation to the subject and is not a complete review. For a comprehensive review of this topic the reader is referred to a review by Miya, Wu and Marcus (1962). Additional summaries covering various aspects of the properdin system have also been published by Rowley (1957), Wedgwood (1959), Chernokhuostova (1960), Homer and McNall (1961), Muschel (1961), and Lepow (1961). Also, a series of articles dealing with properdin may be found in a volume of the Annals of the New York Academy of Sciences (v. St. Whitelock and Furness, 1956).

A. Characteristics of Properdin and the Properdin System.

Properdin is a euglobulin comprising not more than 0.02 per cent of normal serum proteins (Pillemer et al., 1954). The protein resides in Fraction III-1 (Cohn) of serum, which is a beta-2 globulin (Pillemer et al., 1954, and Pillemer, 1956) or a gamma-1 globulin according to current conventions (Lepow et al., 1959). In purified preparations the sedimentation constant of the component primarily associated with properdin activity is 18S (Lepow et al., 1959), although values as high as 27S have also been reported (Pillemer et al., 1954). Its heat stability is dependent on the concentration of properdin, that in untreated serum being inactivated at 56°C in 30 minutes (Pillemer et al., 1954). Activity is present in purified properdin (100-200 units/ml) after heating at 85°C for 30 minutes (Todd,

Pillemer and Lepow, 1959); 100°C, however, causes rapid inactivation of the purified material (Pillemer et al., 1954).

Contrary to the properties of many biological substances the storage of purified properdin is best accomplished in the fluid state at +2°C rather than at -20°C or -70°C. In fact, properdin activity of sterile preparations was maintained for at least 8 months at the higher temperature, while activity began to diminish at about 119 days when stored at -20°C and within 2 days at -70°C (Todd et al., 1959). In contrast to this loss of activity in the purified state, untreated serum may be stored for extended periods at -70°C without loss of properdin activity (e.g. Hinz, Jordan and Pillemer, 1956 and Southam and Pillemer, 1957).

The various activities attributed to properdin require the interaction of several factors. These include properdin, the four components of complement (C'1, 2, 3, and 4), and Mg++, which together comprise the "properdin system" (Pillemer et al., 1954). For example, the reaction of properdin with zymosan (the insoluble residue of yeast cell wall) proceeds in two steps. The first stage involves the combination of properdin with zymosan and requires the presence of Mg++, C'1, C'4 and temperatures between 10°C and 20°C with optimal being 15°C (Pillemer, 1956). The second stage of the reaction involves the inactivation of C'3 by the properdin-zymosan (PZ) complex. This reaction requires Mg++ and temperatures above 20°C, proceeding most rapidly at 37°C (Pillemer et al., 1954). The zymosan titration of properdin is based upon these reactions. One unit of properdin is defined as that amount of the euglobulin which will complex with zymosan and inactivate 120 $\pm$ 30 units of added C'3 under

carefully standardized conditions (Pillemer et al., 1956). Properdin has also been found to complex with other polysaccharides besides zymosan, such as dextran, endotoxins, and mucins (Pillemer, 1956).

Many attributes have been assigned to the properdin system. Among these are bactericidal (Wardlaw and Pillemer, 1956) and virucidal properties (Ginsberg and Wedgwood, 1956). In the former group some six species of gram negative bacteria and one specie of a gram positive bacteria were sensitive to the action of properdin, although the susceptibility within each species varied widely (Wardlaw and Pillemer, 1956). Viruses known to be inactivated are Newcastle disease virus (Wedgwood, Ginsberg and Pillemer, 1956), influenza A and B (Ginsberg and Wedgwood, 1956), herpes simplex virus (Finkelstein, Allen and Sulkin, 1958), and T2 (Van Vunakis, Barlow and Levine, 1956) and T7 bacteriophages (Ginsberg and Wedgwood, 1956).

Other activities of the properdin system include the hemolysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (Hinz et al., 1956). In addition, low properdin levels have been associated with certain bacterial infections such as pneumonia and pyelonephritis (Hinz, 1956); with the failure to reject cancer cell homografts (Southam and Pillemer, 1957); and with radiation injury (Ross et al., 1955, and Ross, 1956), although no beneficial effects were found when this protein was administered to irradiated animals (Miya, Marcus and Thorpe, 1958). Furthermore, the properdin system may be important in histoplasmosis (Miya and Marcus, 1961), coccidioidomycosis, and blastomycosis (McNall, Newcomer and Sterberg, 1959), but not cryptococcosis

(Gadebusch, 1961). Also, decreased levels of properdin during pregnancy may account for decreased resistance of pregnant women to certain infections (Homer and McNall, 1961).

B. The Properdin System in Phagocytosis and Cytopepsis.

The role of the properdin system in phagocytosis and cytopepsis still remains to be elucidated, although these activities were found not to be enhanced by Rutenburg and Fine (1957). These investigators utilized a system of normal rabbit granulocytes and macrophages in (1) normal rabbit plasma, (2) normal rabbit plasma with added properdin, (3) plasma from rabbits subjected to hemorrhagic shock, which was presumably deficient in properdin, and (4) plasma from shocked animals with properdin added to the plasma. The phagocytic and bacteriostatic index of the phagocytes in each of the above menstruums was then measured after addition of Friedlander's bacillus (K. pneumoniae). In no instance did the increased levels of properdin significantly increase the degree of phagocytosis or the bacteriostatic index of the phagocytes. However, it is possible that small amounts of properdin already present in normal rabbit serum and possibly present in "shock plasma" could have given the results that were found, abolishing any effect the added properdin may have given. Wardlaw and Pillemer (1956) have reported that as little as 0.01 unit of properdin showed definite bactericidal effect.

Miya and Marcus (1961) employing P-32 labeled H. capsulatum and mouse peritoneal macrophages also failed to find increased rates of phagocytosis by the presence of properdin. The total amount of protein added to the system, all of which was in the form of purified human

properdin, was very small however, and this lack of protein may have had an inhibitory action on the ingestion process. In further studies with RP reagent (serum lacking properdin) and RP reagent with added properdin phagocytosis by both "immune" and normal macrophages was found to be increased over that where properdin alone was added. The amount of total protein added in these later experiments was greater than when properdin alone was used. Perhaps this could account for the increased rate of phagocytosis, although the presence of complement, which was lacking when properdin alone was used, also could have influenced phagocytic rates. It is interesting to note that in these experiments no differences were found in the rates of phagocytosis between normal and "immune" macrophages.

In the same system Miya and Marcus (1961) also found that properdin may have caused increased rates of cytopepsis by phagocytes from immune animals over controls. The presence of the properdin system, on the other hand, did not give cytopeptic rates by "immune" cells greater than that of normal cells above that found in the presence of RP reagent. However, both of these gave greater differences in cytopeptic rates between the two cell populations than properdin alone. This indicates that perhaps C' is necessary for the observed results. The comparative effects between treatments within a single cell population (i.e. "immune" or normal phagocyte population) were not made here, so although properdin was believed to enhance digestion by "immune" macrophages the amount of enhancement in comparison with that given by the total properdin system or C' alone is not known.

The opsonic index of guinea pig serum was reported by Nanni (1958) to be increased by the presence of properdin. Here S. aureus was incubated with guinea pig leukocytes in (1) normal guinea pig serum, (2) guinea pig serum treated with zymosan at 17°C to remove endogenous properdin with and without human properdin added back to the serum, and (3) heat inactivated guinea pig serum with and without human properdin. By measuring the number of organisms phagocytized after a 20 minute incubation period it was found that the addition of properdin to both heat inactivated and zymosan-treated serum caused increased opsonic activity over that when the protein was absent and the activity was equivalent to that of untreated serum. From these results it appears that complement does not play a significant role in increasing the opsonic index of phagocytes in the presence of properdin since both heat-treated serum, which inactivates C', and serum treated with zymosan at 17°C, which leaves C' intact, had about the same amount of opsonic activity when properdin was added back to each system.

In other studies by Howard and Wardlaw (1958) evidence was presented that properdin might participate in the removal of E. coli and K. pneumoniae by phagocytes of rat livers. It was noted by these investigators that perfusion of fluids containing bacteria through this organ resulted in increased uptake by the phagocytes in the presence of unheated normal rat, mouse or human sera as compared to that with a menstruum of a balanced salt solution. Absorption of the sera with the organism being studied to remove naturally-occurring opsonins caused a reduction in clearance by the perfused liver; however, rates of uptake were still

greater than that obtained in salt solutions alone. Subsequent treatment of the absorbed serum with zymosan to remove properdin but not C' caused a complete loss of the opsonizing capacity of the sera. It thus appeared that properdin might play some role in enhancement of phagocytosis, but results were obscured by the fact that addition of purified properdin to the bacteria and zymosan absorbed sera failed to reactivate the opsonic activity that had been noted with sera that had been absorbed only with bacteria.

Therefore, it is apparent that the role of the properdin system in phagocytosis and cytopepsis remains equivocal.

MATERIALS AND METHODS

The procedures followed here were essentially those originally described by Hill and Marcus (1960) using sodium radiophosphate (P-32) labeled organisms. Certain modifications were introduced in an attempt to simplify the method. The complete procedure and materials employed were as follows in the subsequent sections.

I. Organisms

Histoplasma capsulatum, strain G17M, was used throughout this study, with the exception of the experiment concerned with the determination of P-32 uptake by yeast cells when Candida albicans was employed.

II. Animals

Adult albino mice (Mus musculus) of mixed sex were used as the source of macrophages. All animals were maintained on commercially prepared pelleted feed and water freely provided.

III. Media

A. Candida albicans.

Slant cultures of this organism were maintained on Sabouraud's agar (Difco). To label the organisms with P-32, a liquid medium with the following formulation was used:

Tryptose phosphate broth (Difco)	29.4 g
Yeast extract	4.0
Maltose	10.0
Cystine	0.005
Water	1000 ml

This was autoclaved at 121°C (15 lb. pressure) for 20 minutes, cooled, then 50 units/ml penicillin and 50 ug/ml of streptomycin added.

B. Histoplasma capsulatum.

The yeast phase of this organism was maintained on slants consisting of the following:

Tryptose Phosphate Broth (Difco)	83%
Agar	2%
Human blood	15%
Penicillin	50 u/ml
Streptomycin	50 ug/ml

For isotope labeling, the same culture medium described for labeling C. albicans was employed.

C. Cell Collecting Fluid (CCF) and Cell Maintenance Fluid (CMF).

Earle's balanced salt solution was the base medium employed for collecting, washing and maintaining macrophages and was made up as follows:

Phenol red	0.02 g
NaCl	6.80 g
KCl	0.40 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$NaH_2PO_4 \cdot H_2O$	0.14 g
Glucose	1.00 g
$CaCl_2$	0.20 g
H_2O	1000 ml

This was prepared 10 times concentrated, autoclaved at 121°C for 20 minutes, and stored at 4°C. For use, the concentrated solution was diluted 1:10 with sterile distilled water.

For the collection of mouse peritoneal exudate phagocytes, the following cell collecting fluid (CCF) was used:

Earle's BSS	90%
5% lactalbumin hydrolysate solution . .	10%
Heparin	10 units/ml
Penicillin.	50 units/ml
Streptomycin.	50 ug/ml

The lactalbumin hydrolysate solution was prepared by suspending 5 g lactalbumin hydrolysate (Nutritional Biochemical Corporation) in 95 ml Earle's BSS (5% solution) then autoclaved at 10 pounds pressure for 10 minutes and stored at -70°C. Before use the frozen solution was placed in a boiling water bath long enough to dissolve particulate material.

Heparin (Eastern Chemical Corporation) was prepared 100 times concentrated (1000 units/ml) in physiological saline, autoclaved for 20 minutes, then stored at 4°C until used. Ten units/ml of cell collecting fluid (CCF) was sufficient to prevent the formation of any fibrin clots in the peritoneal exudates after collection.

For washing the phagocytes and maintaining them in vitro the following cell maintenance fluid (CMF) was employed:

Earle's BSS 70%

5% lactalbumin hydrolysate solution 10%

Sterile, heated (56° C/30 min) calf serum . 20%

Penicillin . . . . . 50 u/ml

Streptomycin . . . . . 50 ug/ml

The calf serum was obtained from blood collected at a local slaughter house. The blood was allowed to clot for 24 hours, serum collected, sterilized by Seitz filtration and stored at -70°C. Before use the serum was heated at 56°C for 30 minutes to inactivate heat labile components.

The pH of both the CMF and the CCF was adjusted to 7.2 to 7.3 before use by the addition of the required amount of sterile 5% $NaHCO_3$ containing 0.002% phenol red.

IV. Immunization

A. Vaccine Preparation.

H. capsulatum, strain G17M, was initially passed through mice and then reisolated on antibiotic-containing blood agar plates. The isolate was subsequently transferred to several blood agar slants and after maximum growth at 37°C the yeast phase organisms were harvested in physiological saline solution (PSS), washed 3 times in PSS and resuspended in PSS in an Erlenmeyer flask. Formalin, 10%, was added to the cell suspension until a final concentration of 0.5% was obtained. Incubation of this formalin treated culture at 35.5°C for 24 hours on the slowly shaking platform shaker was sufficient to kill the organisms as evidenced

by the absence of growth on subsequent blood agar culture. The vaccine was stored at 4°C, and diluted to the required concentration with sterile PSS before use.

B. Immunization.

Immunization consisted of giving mice 2 or 3 intravenous injections of the H. capsulatum vaccine, 0.5 ml per injection. Control groups were injected with 0.5 ml PSS at the same times as those receiving the vaccine. The animals were allowed to rest 2 weeks after the final injection when induction of peritoneal exudate macrophages was started.

V. Humoral Factors

A. Antiserum.

As a source of antibody against H. capsulatum rabbit antiserum was used. Rabbits were immunized with the previously described vaccine by giving a series of intravenous injections over a period of 3 weeks. Ten days following the last injection the animals were bled by cardiac puncture, the blood pooled and allowed to clot at room temperature for 2 hours and kept overnight in the refrigerator. The serum was sterilized by Seitz filtration and stored at -70°C. Prior to use, the serum was heated at 56°C for 30 minutes.

B. Complement (C').

Normal guinea pigs were bled by cardiac puncture and the blood pooled. The resulting serum was sterilized by filtration through a Seitz or Millipore filter and stored at -70°C until used. The hemolytic titration for C' was carried out by the micro-Kolmer assay (Kolmer, Spaulding and Robinson, 1951).

C. Complement Reagents.

1. Midpiece and endpiece. These complement fractions were prepared from pooled guinea pig serum by the dialysis method described by Kabat and Mayer (1961). The final volume of M and E reagents was 1:2 in fresh physiological saline solution with respect to the original volume of whole serum employed. The complement component activity of each of these reagents is shown in Table 2. The procedure employed in component titration will be described below.

2. R3. R3 (C' lacking C'3) was prepared by absorbing pooled guinea pig serum for three 1 hour intervals at 37°C with a total of 10 mg of zymosan per milliliter of serum. The preparation of zymosan, lot #9B551 was obtained from Fleischmann Laboratories, New York. Subsequent titration of the serum after this treatment revealed that the hemolytic activity and therefore C'3 was still present.

The apparent explanation for this lack of C'3 inactivation is attributable to the low levels of properdin that are present in guinea pig serum. The presence of the properdin factor is necessary for the inactivation of C'3 (Pillemer et al., 1954). To obviate this situation a procedure modified from that described by McNall (1957) was employed. By this method 10 mg zymosan/ml were suspended in human serum at 17°C for 1 hour. At this temperature the properdin in the serum selectively complexes with zymosan (Pillemer et al., 1954). The complex was washed twice with veronal buffer, pH 7.4 (Kabat and Mayer, 1961), then resuspended in the previously treated guinea pig serum at a concentration of 5 mg/ml and incubated for 1 hour at 37°C with frequent agitation. The

properdin contained in the properdin-zymosan complex was now able to inactivate the remaining C'3 in the serum as shown by hemolytic component titration (Table 2).

3. R4. R4 (complement lacking C'4) was prepared by treating 30 ml guinea pig serum with 6 ml 0.15M hydrazine hydrate solution (obtained from the Matheson Coleman and Bell Division of the Matheson Co., Inc., Norwood, Ohio) for 1 hour at 37°C. Subsequent hemolytic component titration revealed that this treatment was adequate to destroy C'4, although C'2 was also inactivated (Table 2).

All complement reagents as well as whole C' were sterilized by filtration through a Seitz filter before use.

D. Component Titrations of Complement Reagents.

The procedure employed in the determination of the hemolytic levels of C'1, C'2, C'3 and C'4 in the various complement reagents was based on that given by Hegedus and Greiner (1938). By this technique the reagent being titrated was serially diluted from 1:10 to 1:360, although in the case of exceptionally low component titers, a 1:2 dilution was frequently used. Next, 0.2 ml of these dilutions was mixed with 0.2 ml of guinea pig R1 (containing C'2, 3 and 4), human R2 (C'1, 3 and 4), human R3 (C'1, 2, 4) and guinea pig R4 (C'1, 2, 3) to detect the C'1, C'2, C'3 and C'4 levels in the reagent being assayed, respectively. Amboceptor, 2 units in 0.1 ml and 0.1 ml of a 2% sheep red cell suspension were added to each tube. The mixtures were incubated for 30 minutes at 37°C, then read for the "100%" hemolytic end point, which is determined by the level of the limiting component. Results are expressed as the reciprocal of the end

point times a factor of 5, since 1/5 of 1 ml was employed in the titrations, and represents the units/ml of the component titrated (Table 2). It should be added that since a 1:2 dilution of the reagent titrated was the lowest employed, the lower limit of the titration was 10 units/ml where more than 1 component was absent. For the E and R3 reagents where only 1 component was absent, the lower limit of titration was less than 1 unit, as found by lack of hemolysis in the standard micro-Kolmer assay for whole C'.

TABLE 2.

Complement reagent component titers.

Reagent	Component Titer			
	C'1	C'2	C'3	C'4
Midpiece	200	< 10	100	< 10
Endpiece	< 1	50	200	800
R3	400	200	< 1	1600
R4	800	< 10	800	< 10

It is noted that in assaying the C' components of the reagents prepared here, human R2 and R3 were employed as standard titrating reagents. This was due to the lack of standardized guinea pig reagents lacking C'2 or C'3. Since the guinea pig midpiece and endpiece prepared as previously described both lack two components of C', they were inadequate for use as R1 and R2 assay reagents.

Because of this difficulty the following procedure was used to prepare R1 and R2: 10 ml guinea pig or human serum was dialyzed against 800 to 1000 ml acetate buffer (pH 5.5, u = 0.02) for 36 to 48 hours in the refrigerator. The dialyzed material was then centrifuged at 1°C at 4000 rpm (2250 x g) for 1 hour, after which the supernatant fluid (R1) was collected, neutralized with 0.5M NaOH, and made isotonic by addition of a proper amount of 10% NaCl. The precipitate (R2) was washed twice with acetate buffer, then taken up in precooled veronal buffer. It is interesting to note that by this procedure adequate R1 and R2 could be prepared from human serum, while only adequate R1 could be obtained from guinea pig serum.

It now seems apparent that the assay for C' components by the procedure of "limiting components" as employed here may not give a true picture of the actual level of the C' component in question (Kabat and Mayer, 1961; Borsos, Rapp and Mayer, 1961). However, it is felt that the results obtained by the procedure described here provide adequate information for the purposes under investigation.

E. Homologous and Heterologous Sera.

Certain experiments to be described below involved the use of human, calf, rabbit, guinea pig and mouse sera. Each of these were obtained from normal individuals and pooled. Heat inactivation was accomplished by heating at 56°C for 30 minutes. The sera were sterilized by passing through a Seitz filter, except for the human serum which was obtained aseptically.

F. Partially Purified Calf Properdin (PPCP).

PPCP was prepared from once-frozen pooled calf serum by a modification of the cold-ethanol fractionation procedure described by Miya and Marcus (1958), then filter sterilized. The properdin concentration obtained was 80 units per milliliter as determined by the zymosan assay of Pillemer et al. (1956). Electrophoresis of the fractionated serum on cellulose acetate strips showed that it consisted of approximately 94% globulins and 6% albumin. The properdin activity was completely destroyed by heating in 56°C water bath for 1 hour.

VI. Isotope Methods

Phagocytosis and cytopepsis were studied in these experiments through the use of P-32 labeled yeast cells. The determination of the optimal P-32 concentration to be used in labeling these organisms was determined by growing C. albicans in the presence of varying concentrations of P-32 in the form of sodium radiophosphate at 35.5°C on the shaker. After good growth was obtained, the labeled cells were centrifuged and the supernate decanted. The organisms were washed 3 times in PSS and resuspended in a medium consisting of 2% glucose and 0.01% KH_2PO_4 (pH 5.0) plus penicillin and streptomycin. The yeast cells were allowed to "respire" in this medium for 24 hours at 35.5°C on the platform shaker at slow speed in order to rid themselves of easily exchanged P-32. The cells were again washed 3 times in PSS and resuspended to the original volume in PSS. The cell concentration of each culture was determined by direct count and 0.5 ml of each transferred into stainless

steel planchets. The planchet samples were oven dried at 110°C for at least 2 hours and the relative radioactivity of each sample determined in a Nuclear Chicago Gas Flow isotope counter (Model D47).

The results of this determination are given in Figure 1. The optimal concentration of P-32 was found to be between 15 and 20 uc per ml, which is within the range reported by Hill and Marcus (1960) when H. capsulatum was labeled with P-32. In all subsequent experiments 15 uc P-32 per ml was used for labeling yeast cells.

H. capsulatum was labeled in essentially the same manner as described above for C. albicans with the exception that Earle's BSS containing 0.5% glucose with antibiotics was used as the "respiring" medium and all washings of the organism were carried out with Earle's BSS. The washed yeast phase organisms were resuspended in CMF, filtered through sterile glass wool to remove clumps of 5 or more cells, counted in a hemocytometer and diluted to a known concentration with CMF or with the menstruum to be studied.

VII. Macrophage Collection and In Vitro Culture

A. Method I.

On the 14th day following the final immunizing injection a mouse peritoneal exudate was induced by injecting 20 ug of sterile glycogen (Nutritional Biochemical Corporation) contained in 1 ml PSS per mouse IP. After 3 days, 5 to 7 ml CCF were injected into the peritoneal cavity following which the animals were sacrificed. The peritoneal cavity was then opened aseptically and the exudate fluids collected. These were

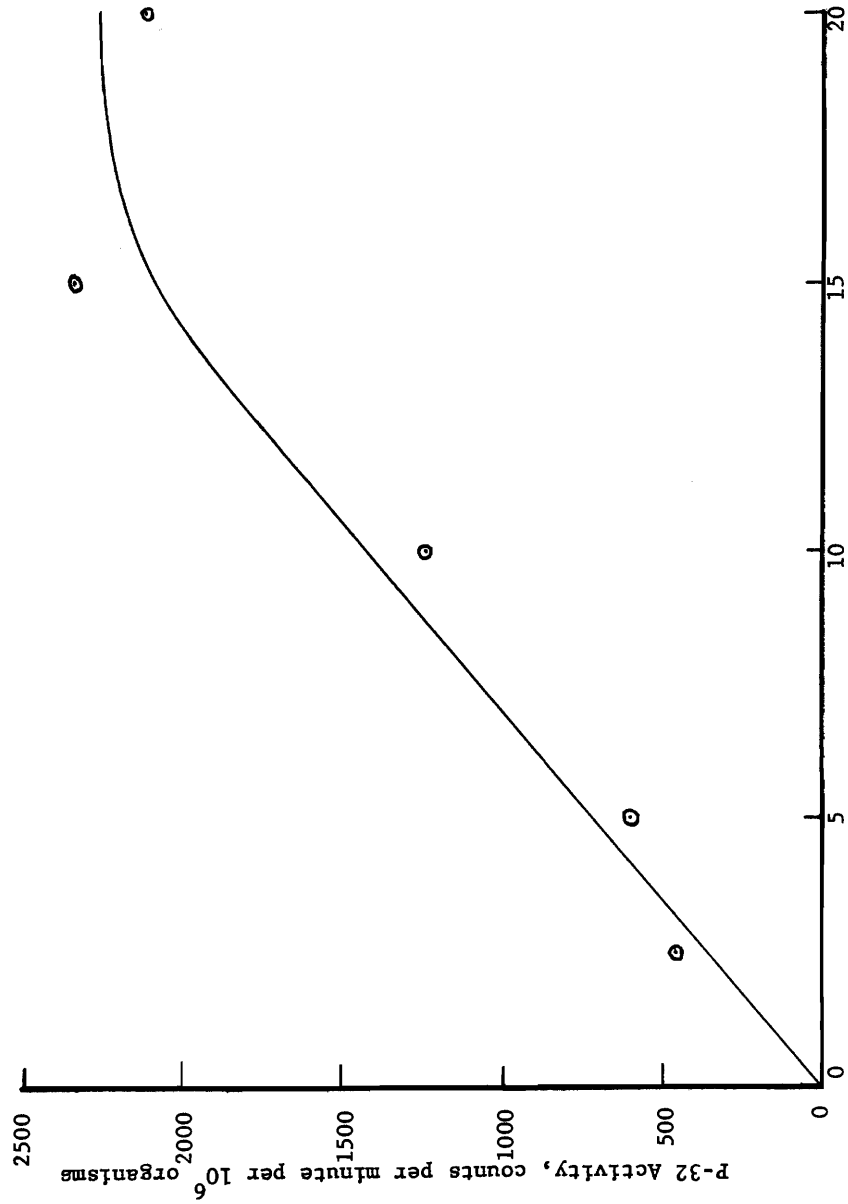

Figure 1. P-32 uptake by Candida albicans.

pooled in sterile siliconed 50 ml centrifuge tubes held in an ice bath, and centrifuged in the cold at 1000 revolutions per minute (approximately 110 x gravity) for 10 minutes to sediment the cells. The supernate was aspirated out and the phagocytes washed once in CMF. The cells were then resuspended in CMF.

The concentration of pooled cells was determined by direct counting in a standard hemocytometer after which they were adjusted to a known concentration with CMF. A constant number of these were next added to 50 ml glass-stoppered flasks and to Pyrex glass planchets containing 18 mm cover slips contained in stendor dishes.

The macrophages were allowed to settle to the bottom and attach to the glass by incubating overnight at 35.5°C without shaking. The suspending medium was then aspirated from the flasks and planchets and the attached cells washed once with fresh CMF.

The next step marked the beginning of the period of phagocytosis. Here, 20 ml of CMF, or the particular menstruum being studied, containing a known concentration of P-32 labeled H. capsulatum was added to each flask or 1 to 2 ml to each coverslip preparation, CMF always being used in the latter case. Immediately after addition and mixing of the suspension, 1 ml was removed from each flask (supernate sample, 0 time of phagocytosis) and placed in stainless steel planchets. Also, at this time a cover slip preparation was removed, washed twice in Earle's BSS, fixed in absolute methanol, then placed in stainless steel planchets and assayed for radioactivity. Afterwards the cover slips were removed, attached to glass slides and stained by the Hotchkiss-McManus procedure

(Periodic acid-Schiff or PAS stain) as reported by Simons (1954). These preparations were examined microscopically for the degree of phagocytosis and digestion. The latter was evidenced by the presence of pink, Schiff-positive material, due to the cytopeptic process of the macrophages.

After all samples had been taken, the flasks and cover slip preparations were incubated at 35.5°C with shaking for 2 to 4 hours during which time supernate and cover slip samples were taken.

After the ingestion period the supernatant fluid was aspirated from the samples, the cells with their ingested organisms washed once with CMF to remove any residual free H. capsulatum, then the original solutions replaced. This period of digestion was followed by again taking supernate and cover slip samples as described above, beginning with 0 time and continuing at various time intervals until termination of the experiment.

All planchet samples were oven dried at 110°C for at least 2 hours to kill the organisms and eliminate the presence of fluids and then the relative radioactivity determined in all samples with a Nuclear Chicago Gas Flow isotope counter (Model D47).

As an additional experimental control, plate counts were run on supernatant samples taken during the course of the experiment. This was done to determine the number of intact H. capsulatum appearing back in the supernatant medium which could alter the results obtained with the isotope experiments. The procedure involved taking a sample of the supernatant fluid from each flask at the same time intervals as that taken for isotope determinations. The samples were diluted in physiological saline and 1 ml of the final dilution plated out on 15% blood agar containing

50 units penicillin and 50 ug streptomycin per milliliter. The plates were sealed with "Parafilm" and incubated at 35.5°C until discrete colonies were plainly visible.

Results obtained from these plate counts indicate that the numbers of H. capsulatum do increase in the supernatant medium with time during the latter phases of the digestion period. However, the increase was insignificant up to and including 32 hours. At the end of 54 hours a significant increase in yeast cells was found, but this was attributed to growth of extracellular organisms and not to release of intracellular histoplasma as evidenced by the condition of the cell culture as determined by both microscopic and gross observations.

B. Method II.

In certain experiments a modification of the above cell culture procedure was employed, although the principle was basically similar. Here, normal and "immune" mouse macrophages were prepared as previously outlined, as were P-32 labeled H. capsulatum. The cell cultures were set up by mixing incomplete cell maintenance fluid (ICMF, consisting of 70% BSS and 10% lactalbumin hydrolysate) with the sera under study in each of several flasks such that a final concentration of 20% serum in the culture medium was obtained. Next, P-32 labeled H. capsulatum were introduced into each flask to give a concentration of 1×10^6 organisms/ml. Macrophages from normal or immune mice were added last, also to give a final concentration of 1×10^6 cells/ml. Immediately after the latter step the contents of each flask were well-mixed and then distributed in 4 ml aliquots to previously silicone-treated screwcapped culture tubes (16 x 150 mm). All

tubes were incubated in a 12 inch diameter roller drum rotating at one cycle every 5 minutes at 35.5°C.

The rate of phagocytosis of H. capsulatum by mouse macrophages was followed by the preparation of smears from the tube cultures at periodic intervals. These were subsequently stained by Wright's method and microscopically examined for ingested fungi. The per cent phagocytosis was based on the number of macrophages containing organisms per 100 phagocytes counted in several random fields.

At the same intervals that smears were taken samples were also collected for isotope assays which were used for studying cytopepsis. These were obtained by centrifuging 4 ml (contents of a culture tube) of each culture (i.e., that containing heated calf serum, unheated calf serum, etc.) at 19,000 rpm (25,000 x g) for 20 minutes to sediment particulate matter. Following this, 1 ml of the supernatant fluids was removed and placed in planchets which were subsequently dried in an oven and assayed for radioactivity.

The hypothesis involved in carrying out this procedure was that cytopeptic activity of the macrophages would cause intracellular release of the P-32 label from H. capsulatum. This radioactivity would then escape into the supernatant fluid and be detected by sampling after removal of extraneous sources of radioactivity by centrifuging the samples at high speeds.

As controls for the procedure, plate counts were carried out on the supernatant fluid to insure the lack of viable labeled organisms. Also, the stability of the label in the yeast phase fungi was assured by

assaying the supernatant activity of cultures lacking phagocytes, but containing labeled organisms.

The method outlined here offers two distinct advantages over that of Method I where the macrophages were allowed to attach to the bottom surface of small flasks. These are: (1) reduced volumes of culture medium required for each variable being studied, and (2) the ability to use the phagocytic cells within a short time (within four hours) after their collection rather than the 24 hour period employed in Method I.

The reduced quantities of culture medium required for each variable is advantageous from the standpoint of requiring less serum, which is very important when the source of this reagent is limited, as in the case of mouse serum. Furthermore, more variables may be studied in any one experiment due to reduced handling of cultures during an experiment.

VIII. Other Materials and Methods

The material and methods outlined above may be considered standard for the experiments to be described. However, in certain instances special procedures or materials were employed. In such cases, these are given in the experimental results in order to maintain continuity.

EXPERIMENTAL RESULTS

I. Phagocytosis and Cytopepsis by Normal and "Immune" Macrophages: Effects of Heated and Unheated Homologous and Heterologous Sera

Many in vitro studies have been carried out which demonstrate that host immunization imparts an enhanced phagocytic and cytopeptic capacity to the phagocytic cells as compared to those from untreated individuals (e.g. Lurie, 1942; Suter, 1953; Donaldson et al., 1956; and Hill and Marcus, 1959). However, it is becoming apparent that operational factors may alter results obtained by different investigators.

The importance of varying an established procedure is brought out by comparing the results of Hill and Marcus (1960) with that of Miya and Marcus (1961). These investigators used essentially the same in vitro procedure, originally described by Hill and Marcus, involving the use of P-32 labeled H. capsulatum to study rates of phagocytosis and cytopepsis by peritoneal macrophages from normal and immune mice. The latter researchers, employing a menstruum containing heated (56°C) calf serum, observed enhanced cytopeptic rates by "immune" cells over those from normal animals. Conversely, Miya and Marcus (1961) were able to demonstrate such a difference in cell populations only in the presence of heat labile factors, such as complement or properdin. In this latter study, however, rabbit or guinea pig sera were used in the culture medium

pointing out the possibility that the source of serum may be important.

This latter point was recently studied by McElree and Downs (1961), who determined the effects of heated sera from various sources on the amount of ingestion of Pasteurella tularensis by rat macrophages. Their findings indicated that the ingestion process is stimulated by the presence of guinea pig and calf serum, but not by horse or rat serum. An interesting note in these experiments was that rat serum was toxic for the rat phagocytes.

The purpose of the present study was to attempt to delineate the effects of homologous and heterologous sera on rates of phagocytosis and digestion of H. capsulatum by normal and "immune" mouse phagocytes in vitro. Also, the added variables of heated and unheated sera were studied to compare the effects of the presence and absence of heat labile factors such as complement and properdin.

A. Effects of Heated and Unheated Homologous and Heterologous Sera on Phagocytic Rates by Normal and "Immune" Mouse Phagocytes.

Peritoneal exudates were obtained in cell maintenance fluid (ICMF + 20% heated calf serum) containing heparin (10 units/ml) from groups of 25 normal and 25 H. capsulatum immunized mice. The macrophages were lightly centrifuged (1000 rpm) for 10 minutes, washed once in cell maintenance fluid (CMF), then resuspended in Earle's BSS. The concentrations of normal and "immune" cells were determined by hemocytometer counts and then adjusted to equal numbers per milliliter by addition of BSS.

Next, several Erlenmeyer flasks were set up and to each were added 40 ml ICMF and 10 ml of one of the sera to be studied (i.e. heated or

unheated human, calf, rabbit, guinea pig and mouse sera) as outlined in Table 3. Following this, P-32 labeled H. capsulatum cells were added to a concentration of 1 x 10^6/ml, then finally, the normal or "immune" macrophages were added, also at a concentration of 1 x 10^6 cells/ml. After mixing the flask contents, 4 ml aliquots were transferred to each of 5 culture tubes which were incubated in a roller drum at 35.5°C. Smears were prepared at 12 hour intervals from each culture containing one of the serum variables, as described under "Materials and Methods," and microscopically examined. Samples obtained during the first 24 hours were evaluated for the degree of phagocytosis by counting the number of macrophages containing H. capsulatum per 100 cells in several random fields. The results of these observations are given in Table 4.

As can be seen from Table 4, the per cent ingestion at 12 hours was slightly higher with the normal cells than the "immune" group, but this is not statistically significant due to the high degree of variability between smear samples. After 24 hours the average difference between the two cell populations was still maintained.

Heating the sera to 56°C for 30 minutes to inactivate heat labile factors did not appear to cause any differences in rates of ingestion by the phagocytic cells. Furthermore, the presence of heated and unheated heterologous and homologous sera did not bring out distinguishable differences in uptake between or within normal and "immune" macrophage populations.

TABLE 3

Culture fluids employed for determination of the effect of heated and unheated homologous and heterologous sera on phagocytosis and cytopepsis by normal and "immune" mouse macrophages.

Serum Treatment	Menstruum
Heated*	Incomplete CMF** + 20% Human Serum
	" " + 20% Calf "
	" " + 20% Rabbit "
	" " + 20% Guinea Pig Serum
	" " + 20% Mouse Serum
Unheated	Incomplete CMF + 20% Human Serum
	" " + 20% Calf "
	" " + 20% Rabbit "
	" " + 20% Guinea Pig Serum
	" " + 20% Mouse Serum

* Heated at 56°C for 30 minutes.

** 70% Earle's BSS + 10% lactalbumin hydrolysate solution.

TABLE 4

Effects of heated and unheated homologous and heterologous sera on phagocytic rates by normal and "immune" mouse phagocytes.

Phagocytes	Sera		Per Cent Phagocytosis*	
	Source	Treatment	12 hours	24 hours
Immune	Human	Heated**	45	42
	Calf		34	47
	Rabbit		38	40
	Guinea Pig		34	50
	Mouse		39	48
	Human	Unheated	44	44
	Calf		36	46
	Rabbit		38	48
	Guinea Pig		37	39
	Mouse		40	50
Normal	Human	Heated	46	57
	Calf		44	48
	Rabbit		38	52
	Guinea Pig		48	58
	Mouse		46	45
	Human	Unheated	40	48
	Calf		44	47
	Rabbit		44	55
	Guinea Pig		48	55
	Mouse		36	52

* Based on the number of phagocytes containing H. capsulatum/100 cells counted.

** 56°C for 30 minutes.

B. Effects of Heated and Unheated Homologous and Heterologous Sera on Cytopeptic Rates by Normal and "Immune" Mouse Phagocytes.

Cytopeptic rates of P-32 labeled H. capsulatum infected normal and "immune" macrophages were followed by radioisotope measurements of the supernatant fluids of the various cultures. This was carried out by centrifuging the entire contents of one set of culture tubes containing each of the serum variables at 0 time and 12 hour intervals thereafter. One milliliter of each supernatant was then placed in a planchet, oven dried, and subsequently assayed for P-32 activity.

The design of this experiment allows several comparisons to be made with regard to cytopepsis by the two cell populations. These are: (1) comparative rates of digestion between normal and "immune" mouse peritoneal macrophages in the presence of heated or unheated human, calf, rabbit, guinea pig, or mouse serum; (2) comparison of the effects of heating of homologous and heterologous sera on either normal or "immune" cell populations; and (3) the relative effects of all the heated or unheated sera on digestion by either normal or "immune" macrophages. To facilitate the presentation of results each of these categories will be discussed separately.

1. Comparative rates of digestion between normal and "immune" mouse phagocytes in the presence of heated or unheated human, calf, rabbit, guinea pig or mouse serum. The release of P-32 from labeled H. capsulatum by intracellular digestion by normal and "immune" mouse macrophages was followed for a period of 60 hours. This was accomplished by sampling the supernatant fluid of centrifuged aliquots of the various menstruums

containing heated or unheated human, calf, rabbit, guinea pig or mouse serum (see Table 3) at 12 hour intervals. The results were then compared for relative rates of cytopepsis of normal and "immune" macrophages in the presence of one of the heated or unheated sera employed in the menstruums. Figures 2 through 11 and Table 5 summarize the results obtained by these comparisons.

Attention to the Figures, where counts per minute (cpm) of P-32 activity are plotted against time reveal several interesting points. First, the "immune" cells in the presence of heated sera (Figures 2 through 6), except that in calf serum (Figure 3) showed an increased rate of cytopepsis over normal cells. This increase was especially noticeable in the presence of heated mouse (Figure 6) and guinea pig (Figure 5) serum where the difference is significantly greater after 24 and 36 hours of incubation, respectively. Significance was based on $\pm$ 2 standard counting errors. Rates of cytopepsis in the presence of heated human (Figure 2) and rabbit serum (Figure 4) were also enhanced in the "immune" cell population but the difference was not as marked as with mouse (Figure 6) and guinea pig serum (Figure 5). The two phagocyte populations suspended in heated calf serum (Figure 3) failed to show any differences in digestive rates.

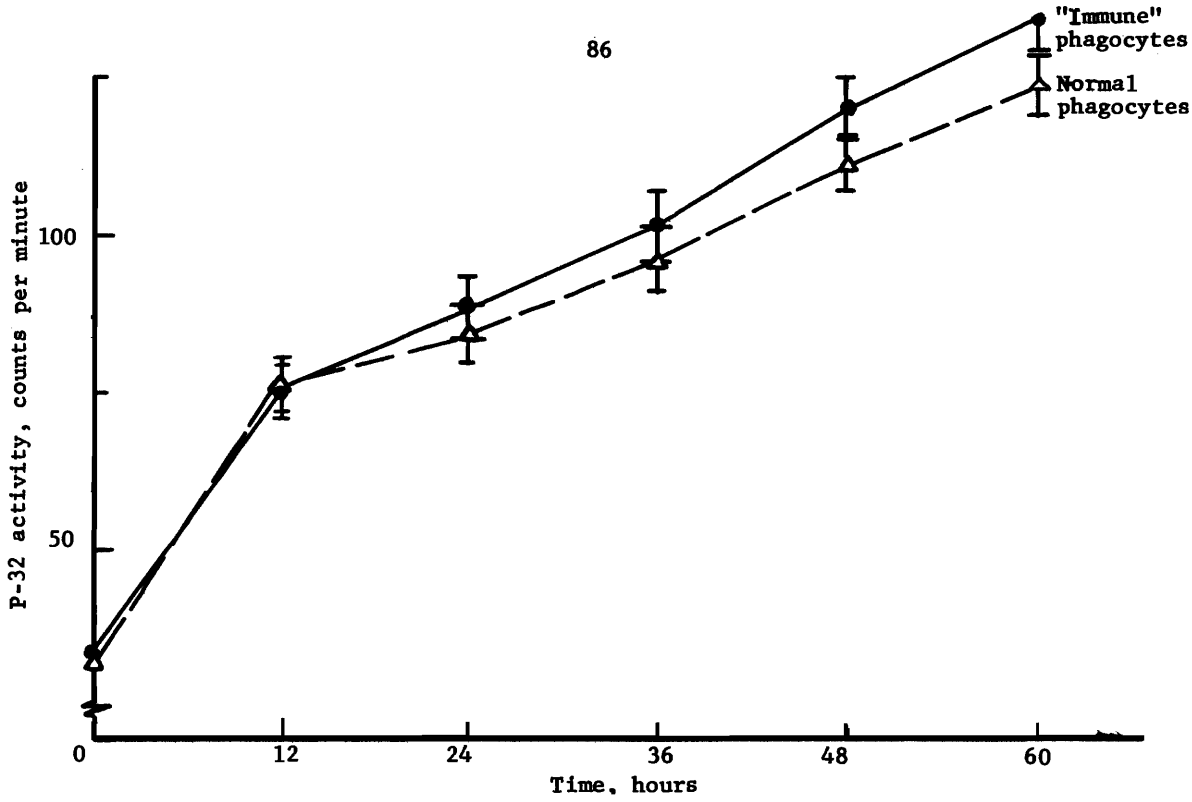

Figure 2. Relative rates of cytopepsis of H. capsulatum by normal and "immune" macrophages in the presence of heated human serum.

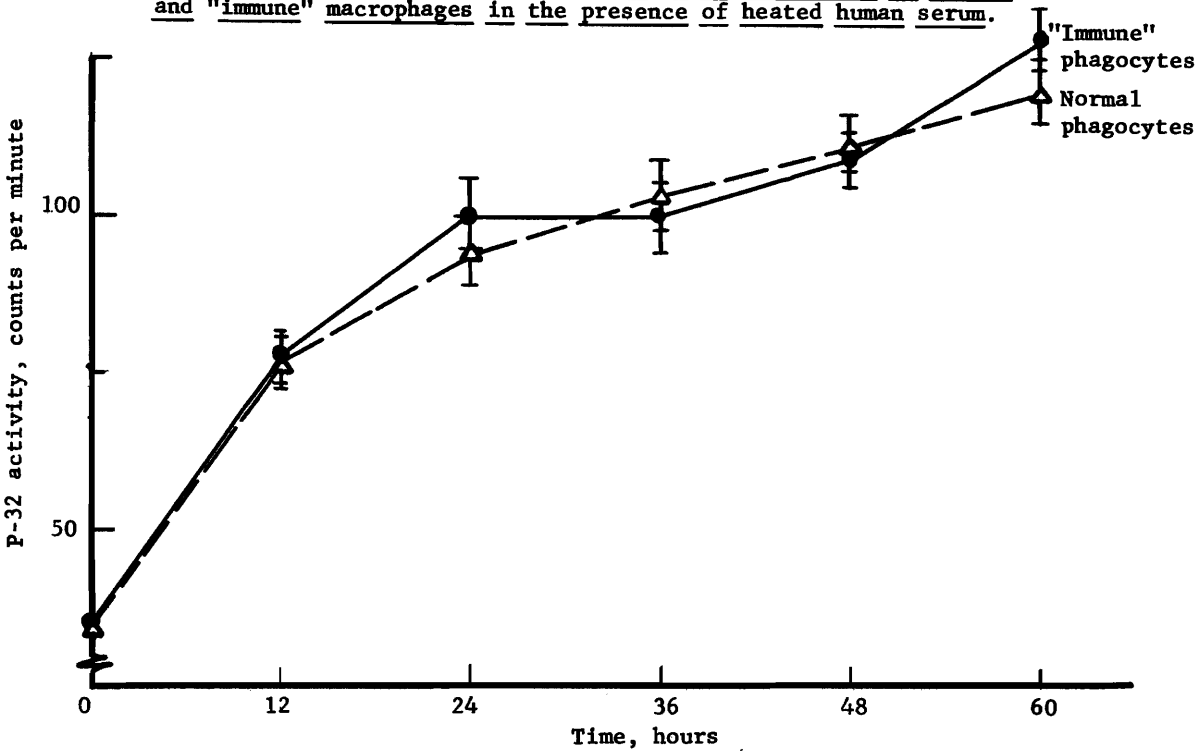

Figure 3. Relative rates of cytopepsis of H. capsulatum by normal and "immune" macrophages in the presence of heated calf serum.

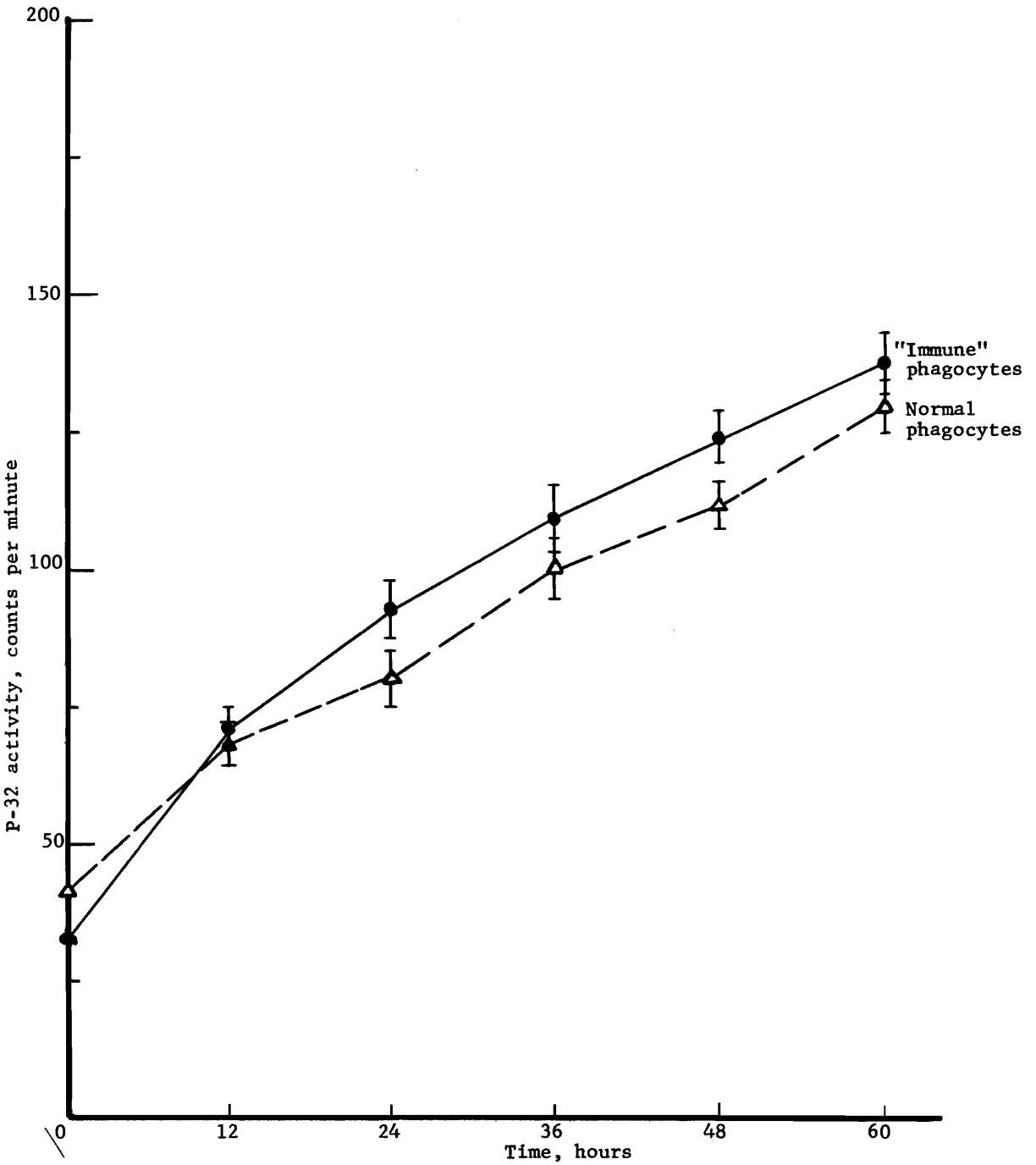

Figure 4. Relative rates of cytopepsis of H. capsulatum by normal and "immune" macrophages in the presence of heated rabbit serum.

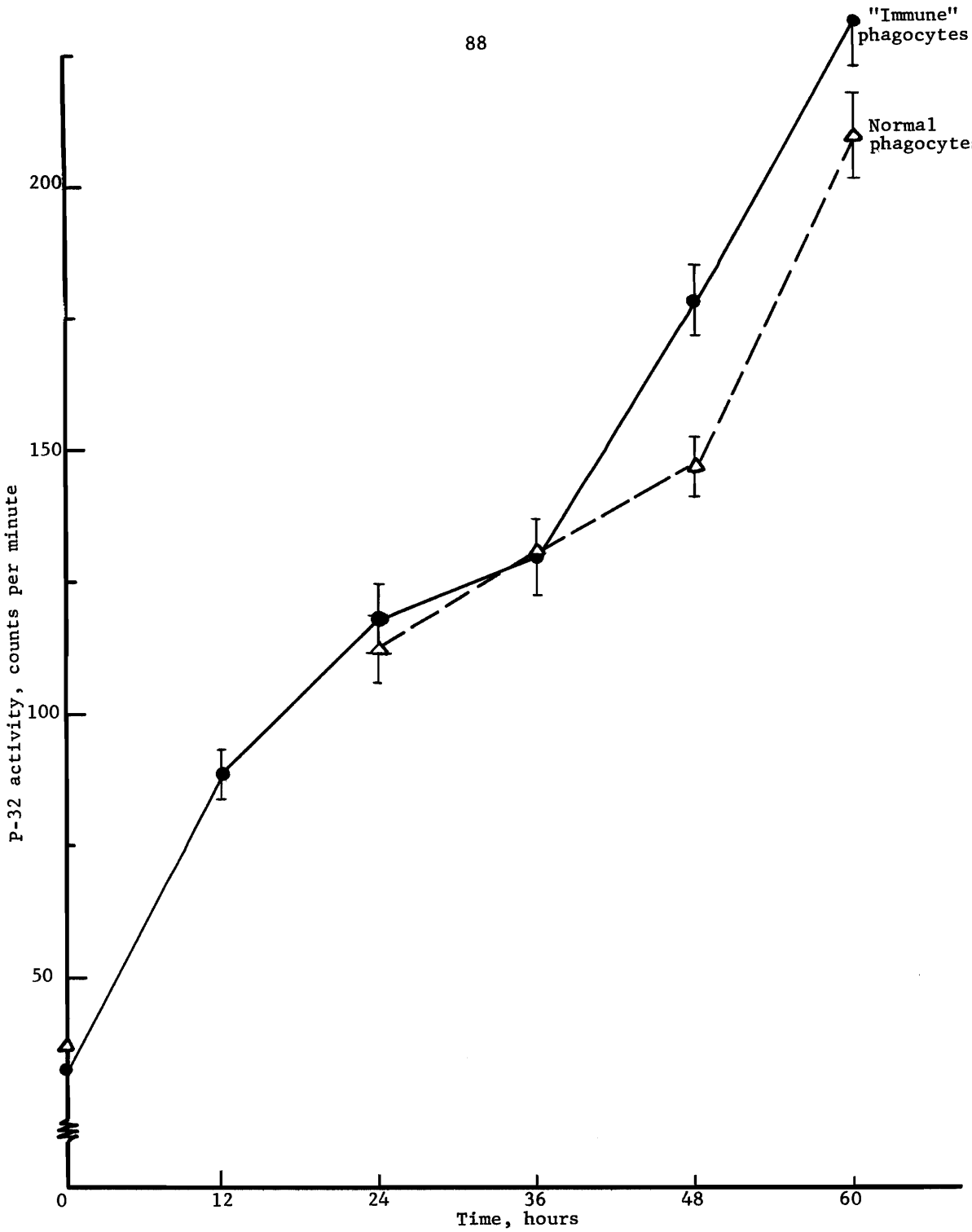

Figure 5. Relative rates of cytopepsis of H. capsulatum by normal and "immune" macrophages in the presence of heated guinea pig serum.

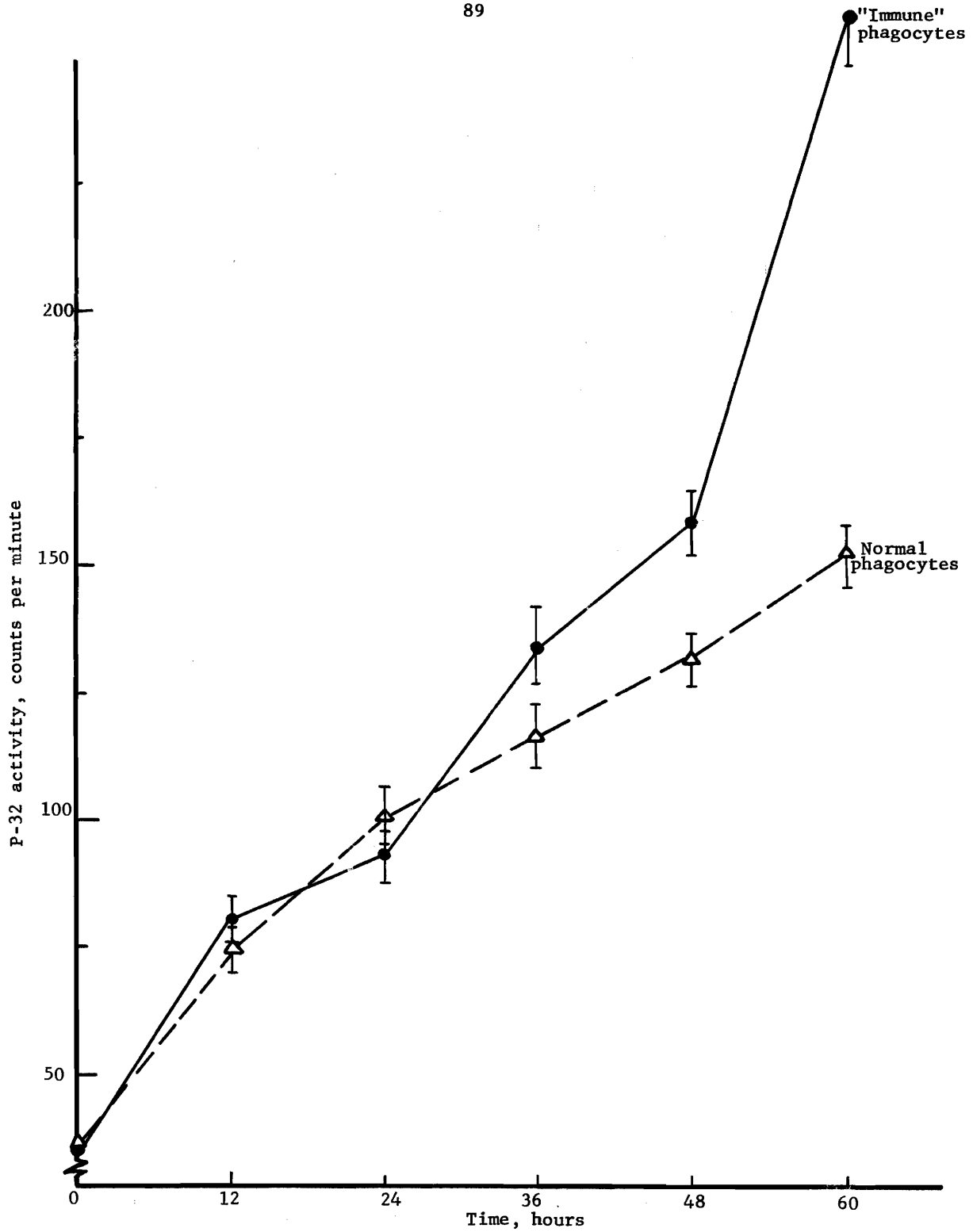

Figure 6. Relative rates of cytopepsis of H. capsulatum by normal and "immune" macrophages in the presence of heated mouse serum.

In the presence of unheated sera (Figures 7 through 11) the differences in normal and "immune" phagocyte activity was not as prominent as in the presence of heated sera. However, the trend of increased cytopepsis by "immune" cells was seen, especially with unheated calf serum (Figure 8) and to a lesser extent with human serum (Figure 7) after 36 hours of incubation. Unheated rabbit (Figure 9) and mouse serum (Figure 11) gave similar results. Significant differences between the latter two cell populations appeared as early as 12 hours and 24 hours, respectively. Unheated guinea pig serum (Figure 10) caused similar increases only after 36 hours. By 60 hours the rates appeared to have fallen to the level of normal cell activity, although this may have been attributable to counting error.

Table 5 summarizes the results of Figures 2 to 11 in terms of positive or negative differences between cytopeptic rates of normal and "immune" cells. Positive differences are used here to indicate significantly higher rates of digestion by the "immune" cells over that by normal phagocytes while negative differences indicate a reverse situation.

It can be seen that in only two cases did negative differences occur, these being in the presence of unheated guinea pig serum at the 24 hour sampling period and with unheated rabbit serum at the 36 and 48 hour intervals. All other periods designated exhibited, to a highly significant degree, more rapid rates of digestion by "immune" cells than normal cells.

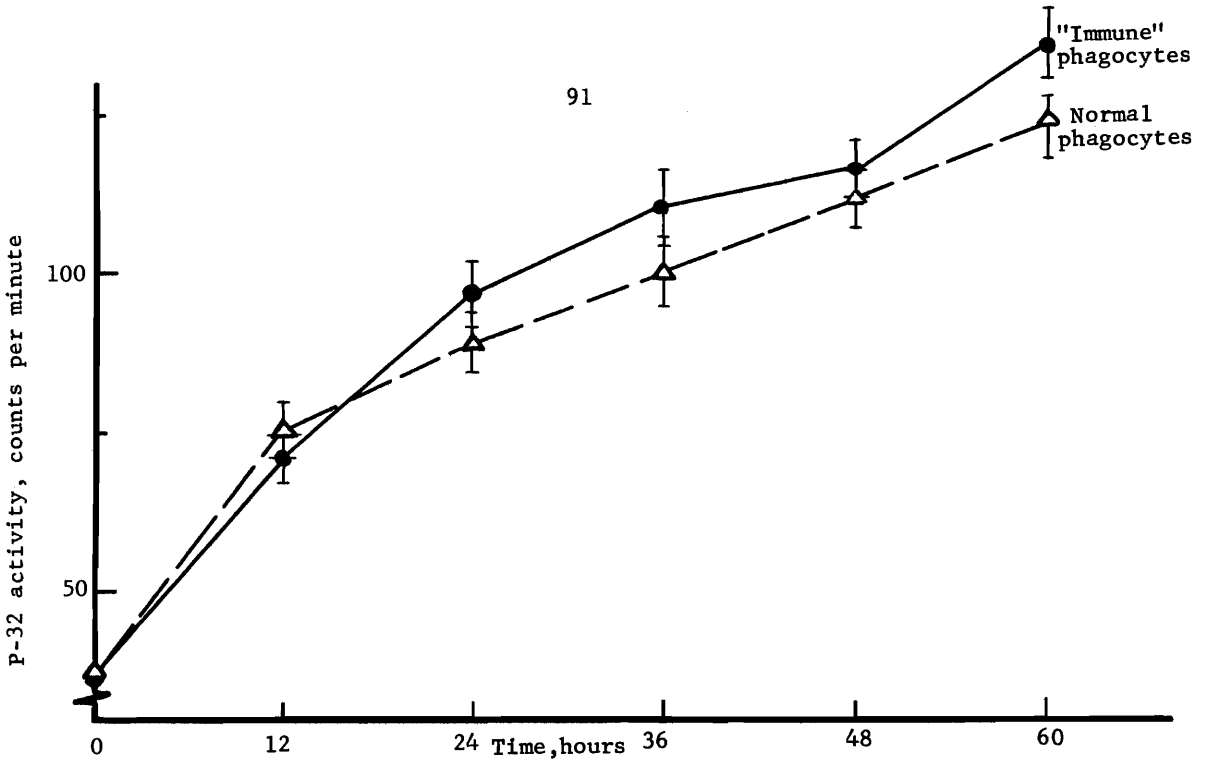

Figure 7. Relative rates of cytopepsis of H. capsulatum by normal and "immune" macrophages in the presence of unheated human serum.

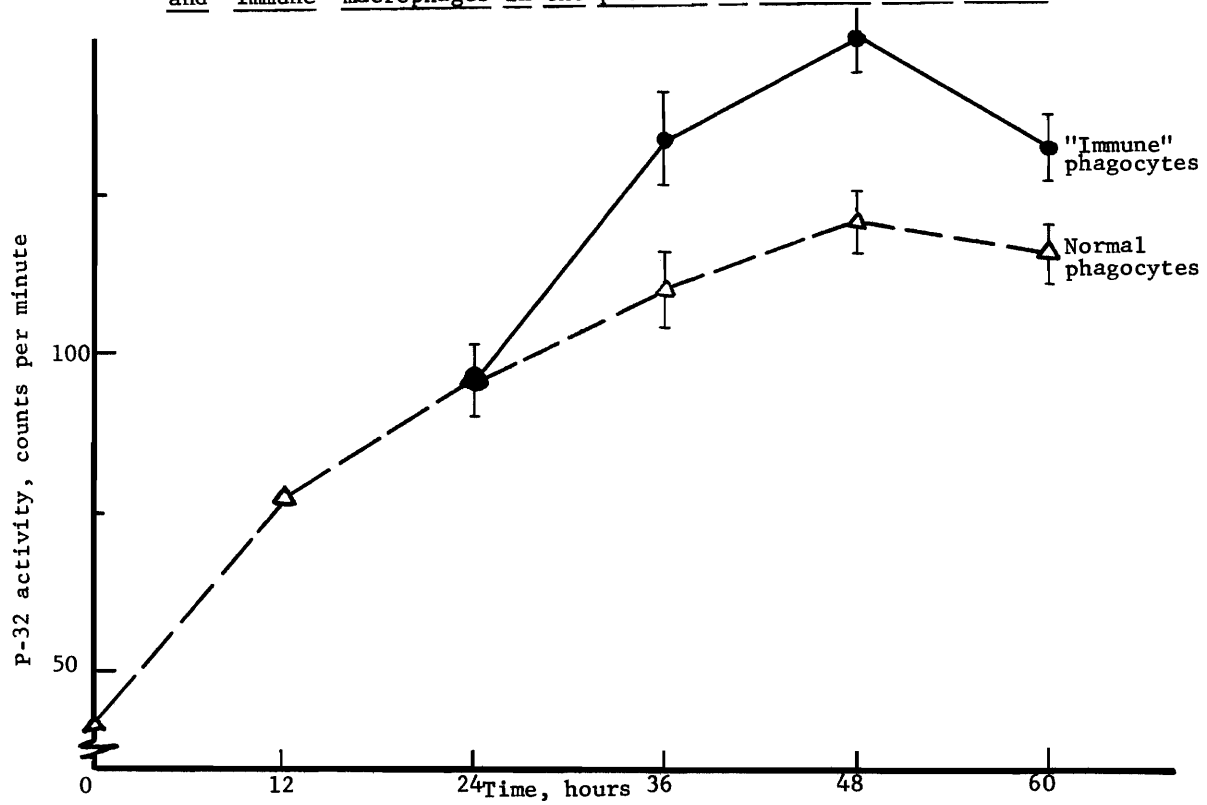

Figure 8. Relative rates of cytopepsis of H. capsulatum by normal and "immune" macrophages in the presence of unheated calf serum.

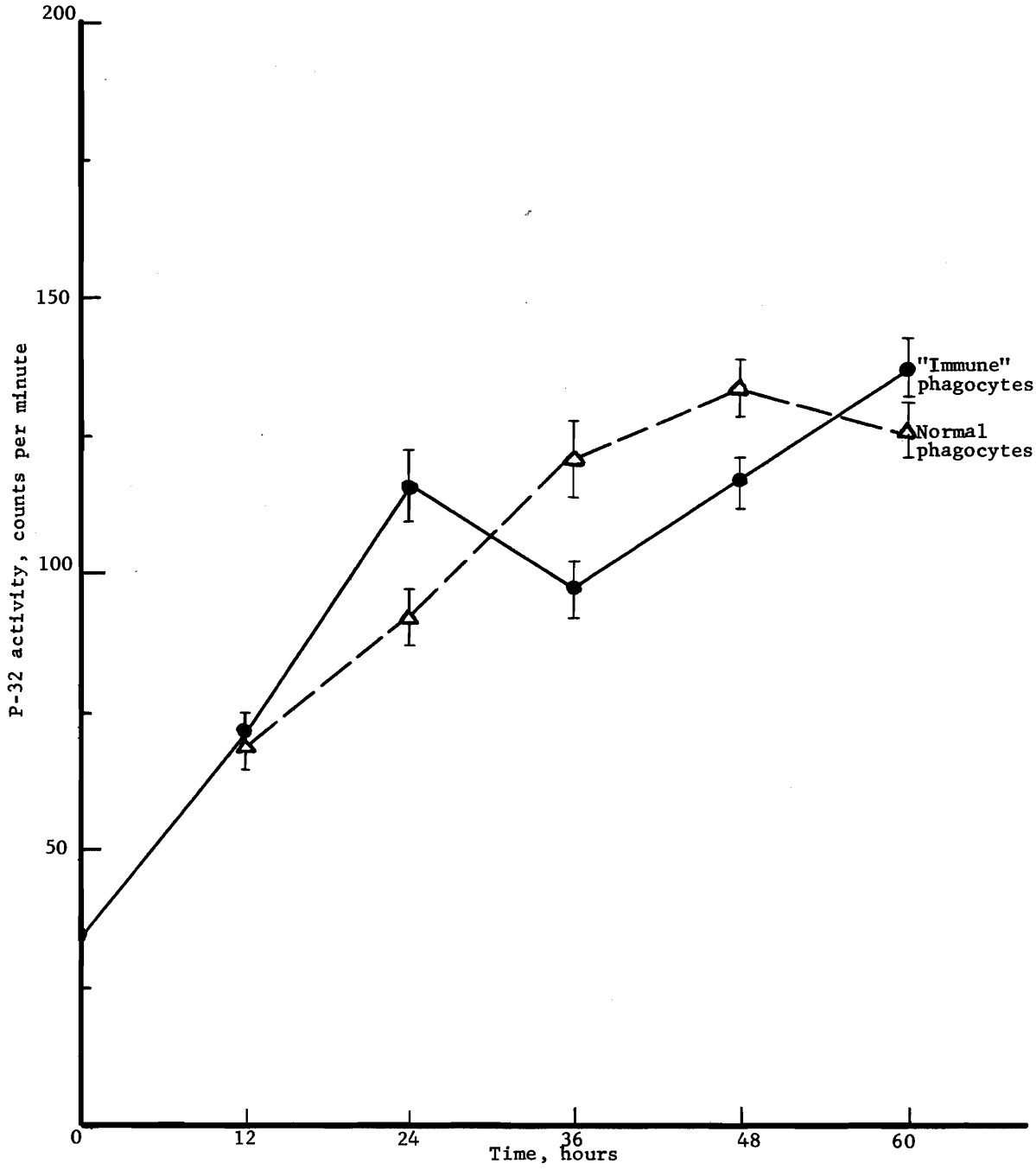

Figure 9. Relative rates of cytopepsis of H. capsulatum by normal and "immune" macrophages in the presence of unheated rabbit serum.

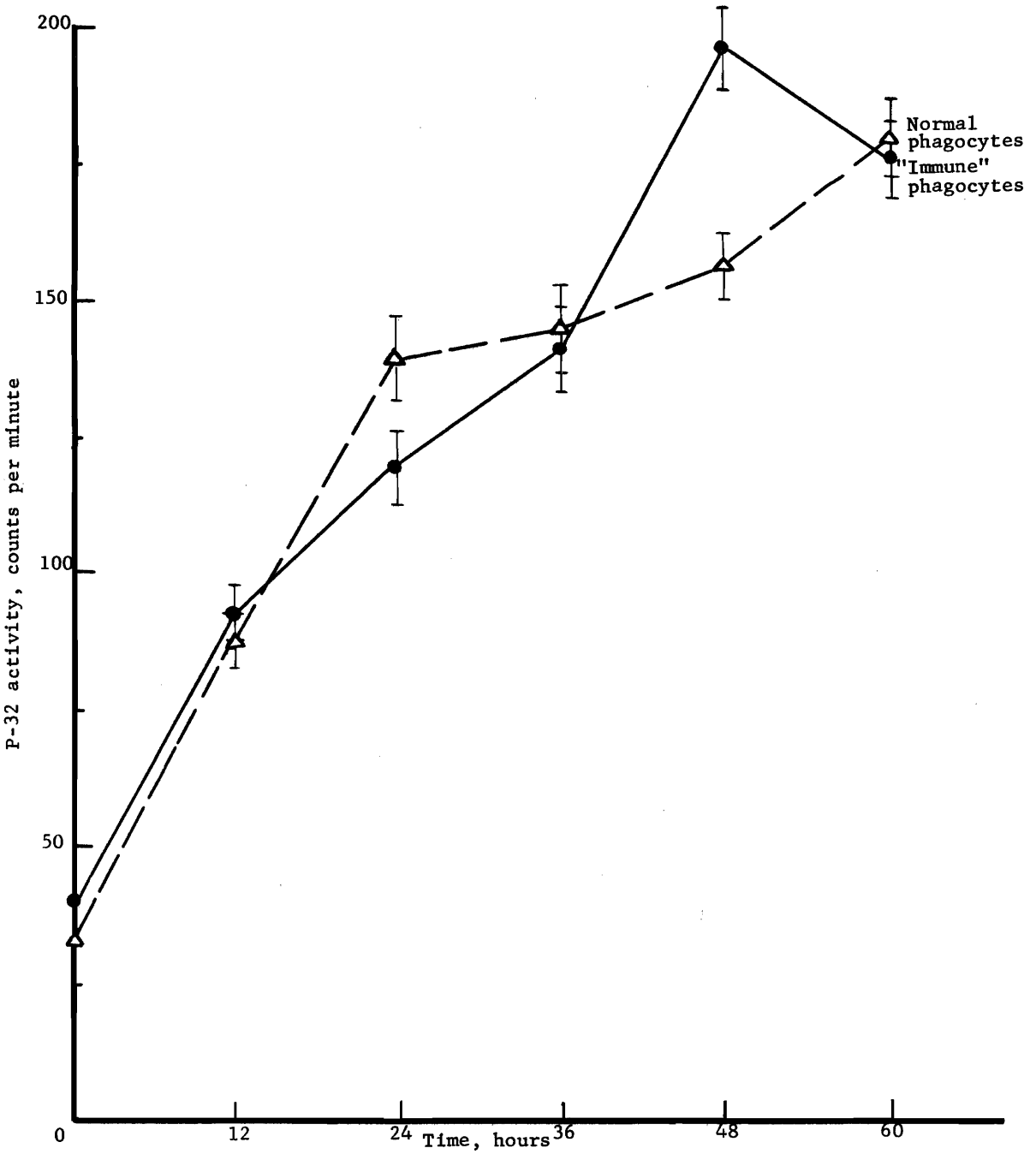

Figure 10. Relative rates of cytopepsis of H. capsulatum by normal and "immune" macrophages in the presence of unheated guinea pig serum.

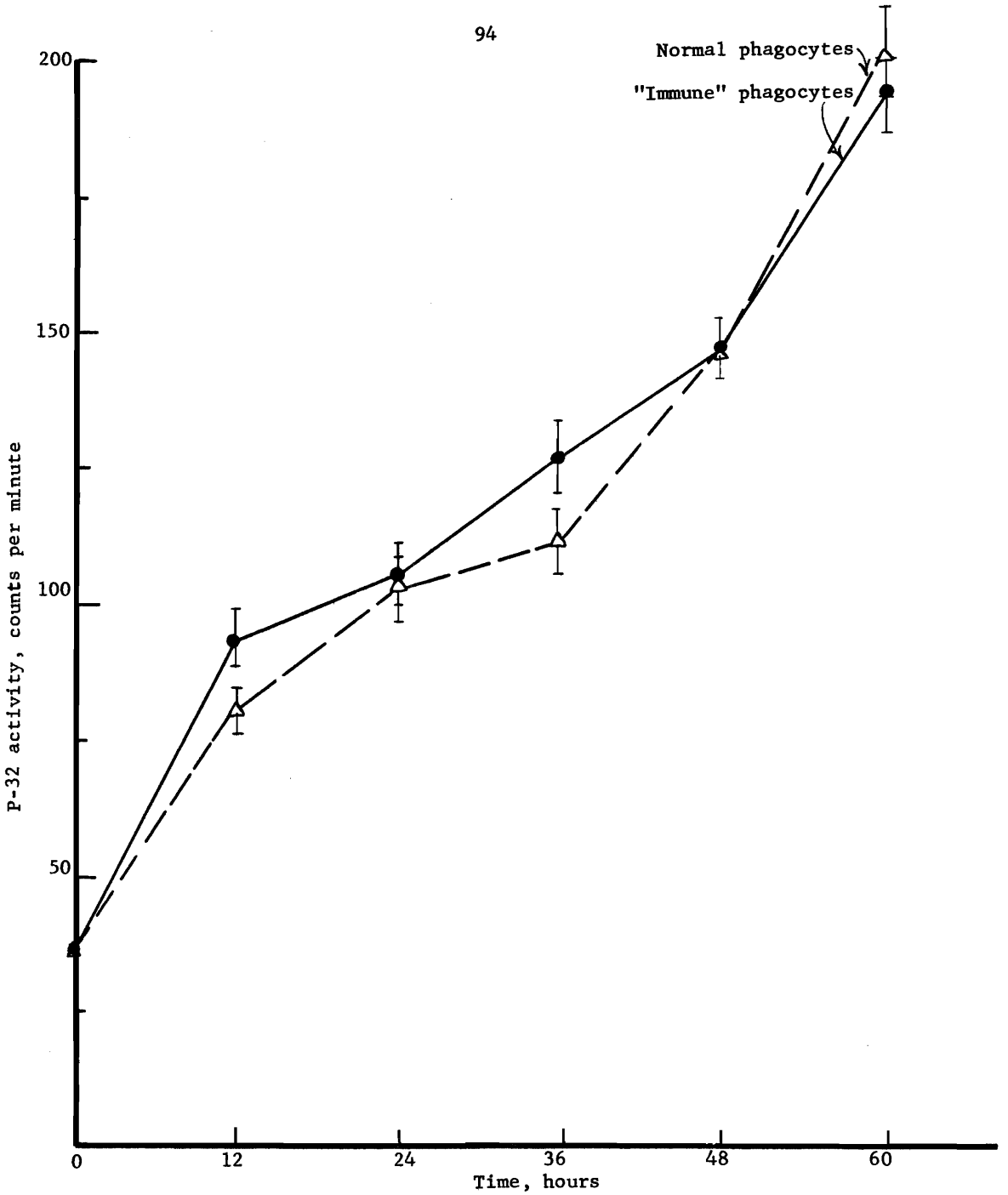

Figure 11. Relative rates of cytopepsis of H. capsulatum by normal and "immune" macrophages in the presence of unheated mouse serum.

TABLE 5

Summary of results comparing the rates of digestion of P-32 labeled H. capsulatum by normal and "immune" mouse phagocytes in the presence of heated and unheated homologous and heterologous sera.

Serum	Serum Treatment	Comparison of "Immune" vs. Normal Phagocytes	
		Positive Difference* (at Sample Time)	Negative Difference** (at Sample Time)
Human	Heated	48 - 60 hours	None
Calf		None	"
Rabbit		24 and 48 hours	"
Guinea Pig		48 - 60 hours	"
Mouse		48 - 60 hours	"
Human	Unheated	60 hours	None
Calf		36 - 60 hours	"
Rabbit		24 and 60 hours	36 - 48 hours
Guinea Pig		48 hours	24 hours
Mouse		12 and 36 hours	None

* Significantly higher rate of digestion by "immune" phagocytes over normal phagocytes (highly significant = $\pm$ 2 standard counting errors).

** Significantly higher rate of digestion by normal phagocytes over "immune" phagocytes.

These results show that under the experimental conditions employed "immune" macrophages were generally more capable of intracellular digestion of the H. capsulatum organisms than were the normal cells. This finding is in agreement with results of other investigators (Hill and Marcus, 1960; Miya and Marcus, 1961).

2. Effect of heating of homologous and heterologous sera on cytopepsis by normal and "immune" mouse phagocytes. The previous analysis did not permit a comparison of the effects of heating each serum on cytopepsis within a single cell population. Therefore, the data were rearranged to reveal these relations. Once again, data were plotted in cpm versus sampling time, but in this instance effects of heated and unheated serum on a single cell population were compared. These comparisons are shown in Figures 12 through 21.

Heat labile factors present in the unheated sera caused a trend toward increased cytopepsis by "immune" phagocytes (Figures 12 through 16). This trend was greatest with calf (Figure 14) and mouse (Figure 16) serum, less so with guinea pig (Figure 15) and rabbit serum (Figure 13), and absent in the presence of human serum (Figure 12). It should be noted that the effect of heat labile factors was transient, usually occurring prior to, or at the 48 hour sampling period. After this time the amount of cytopepsis by "immune" cells was observed to be no greater or even less than that in the presence of heated sera. One obvious explanation for this phenomenon is that the labile factors may become inactivated during incubation after which rates of cytopepsis decrease.

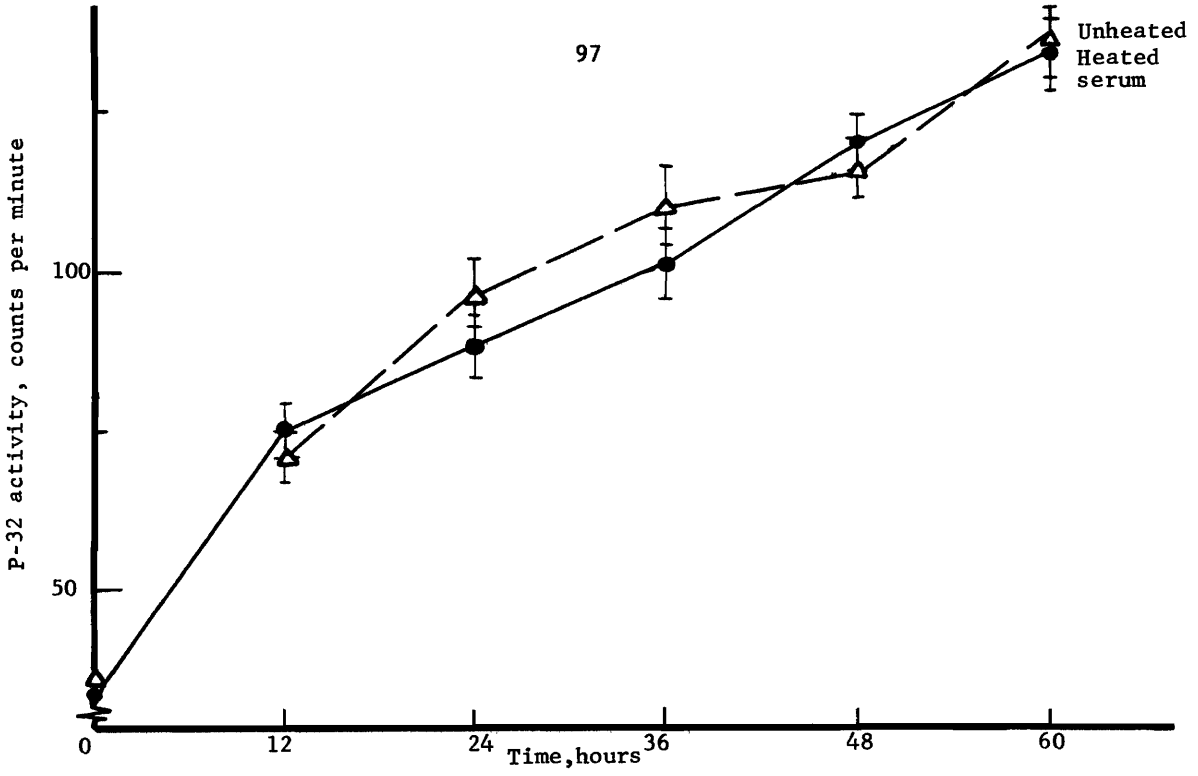

Figure 12. Relative rates of cytopepsis of H. capsulatum by "immune" macrophages in the presence of heated and unheated human serum.

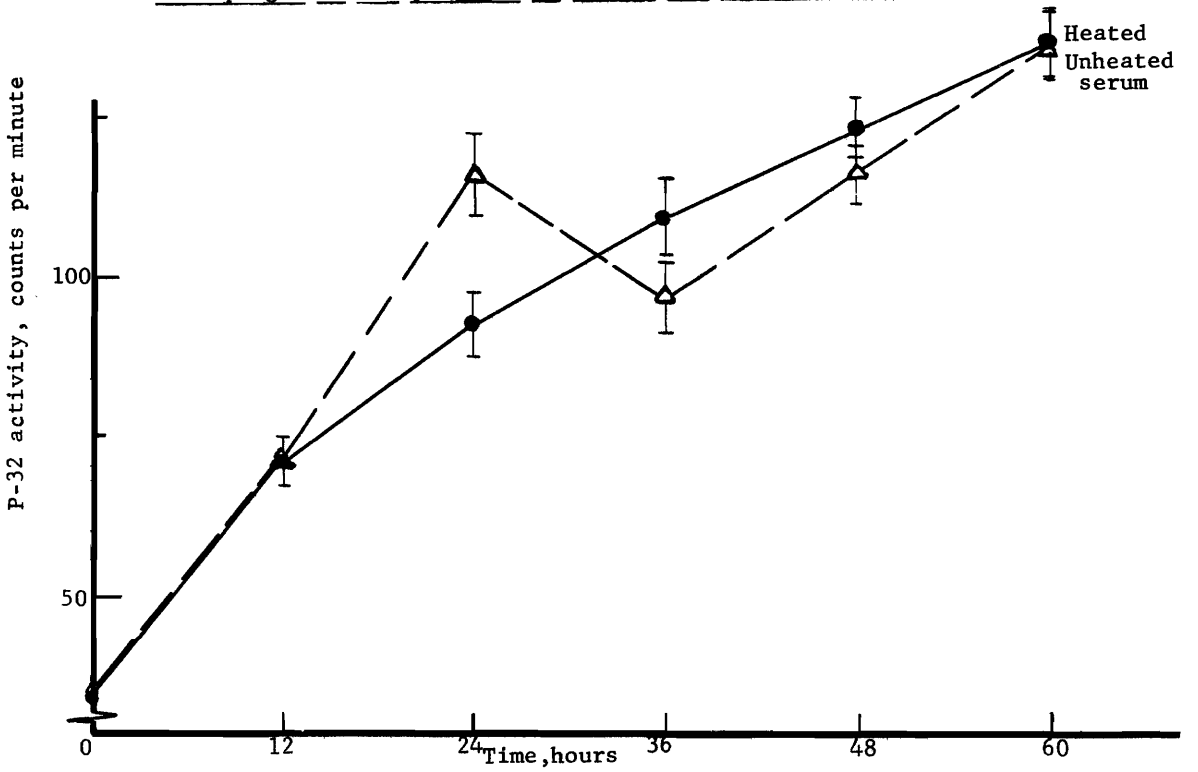

Figure 13. Relative rates of cytopepsis of H. capsulatum by "immune" macrophages in the presence of heated and unheated rabbit serum.

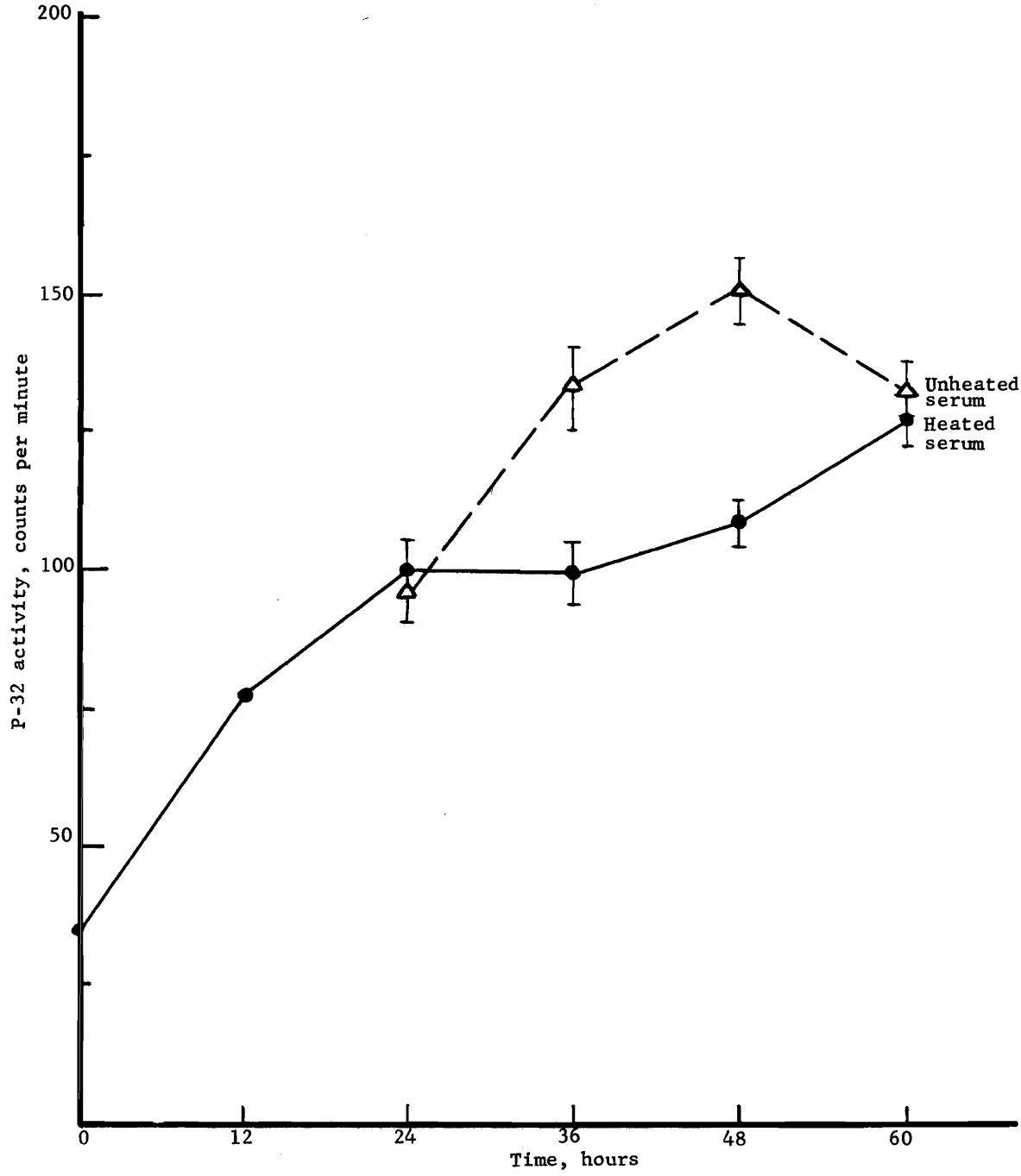

Figure 14. Relative rates of cytopepsis of H. capsulatum by "immune" macrophages in the presence of heated and unheated calf serum.

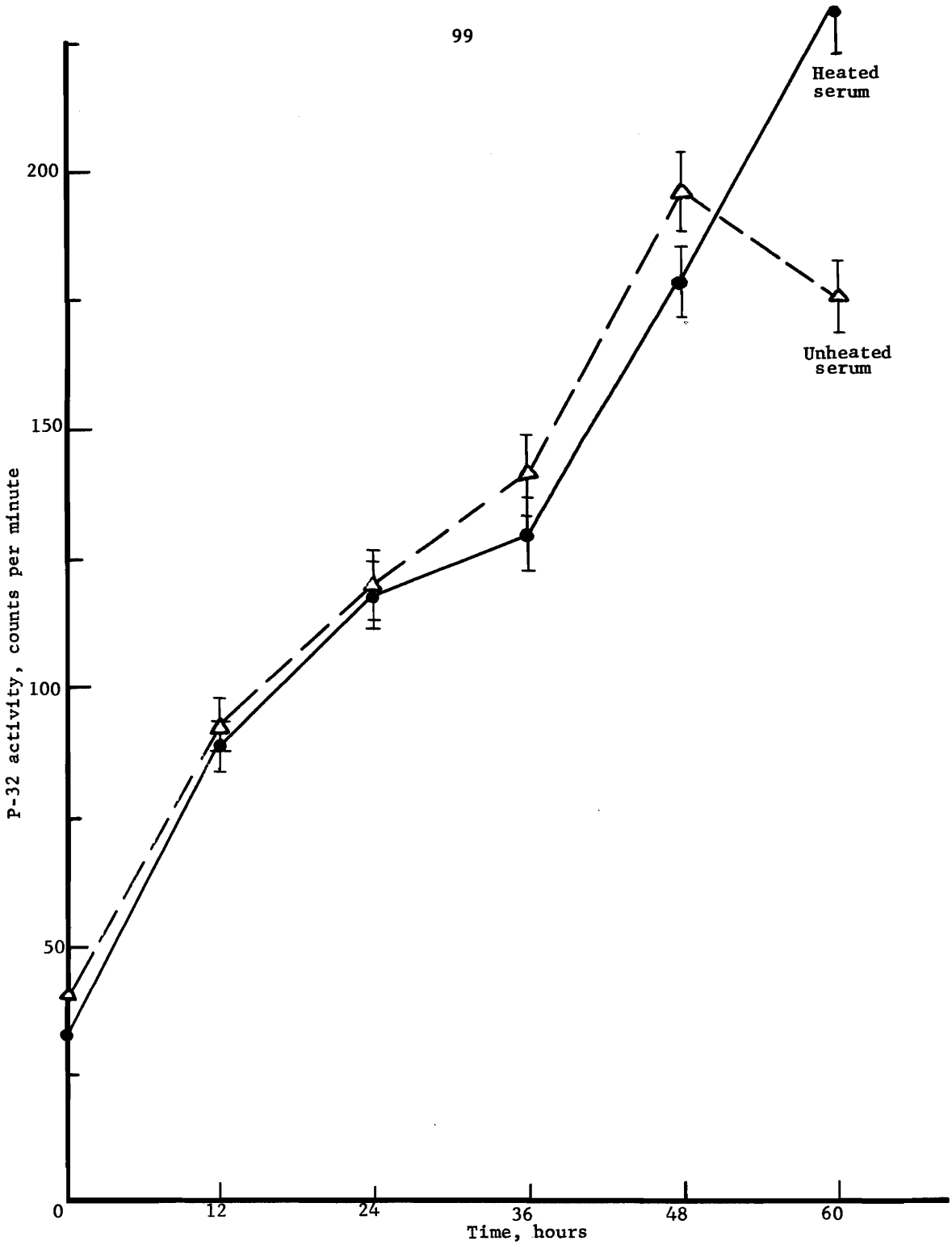

Figure 15. Relative rates of cytopepsis of H. capsulatum by "immune" macrophages in the presence of heated and unheated guinea pig serum.

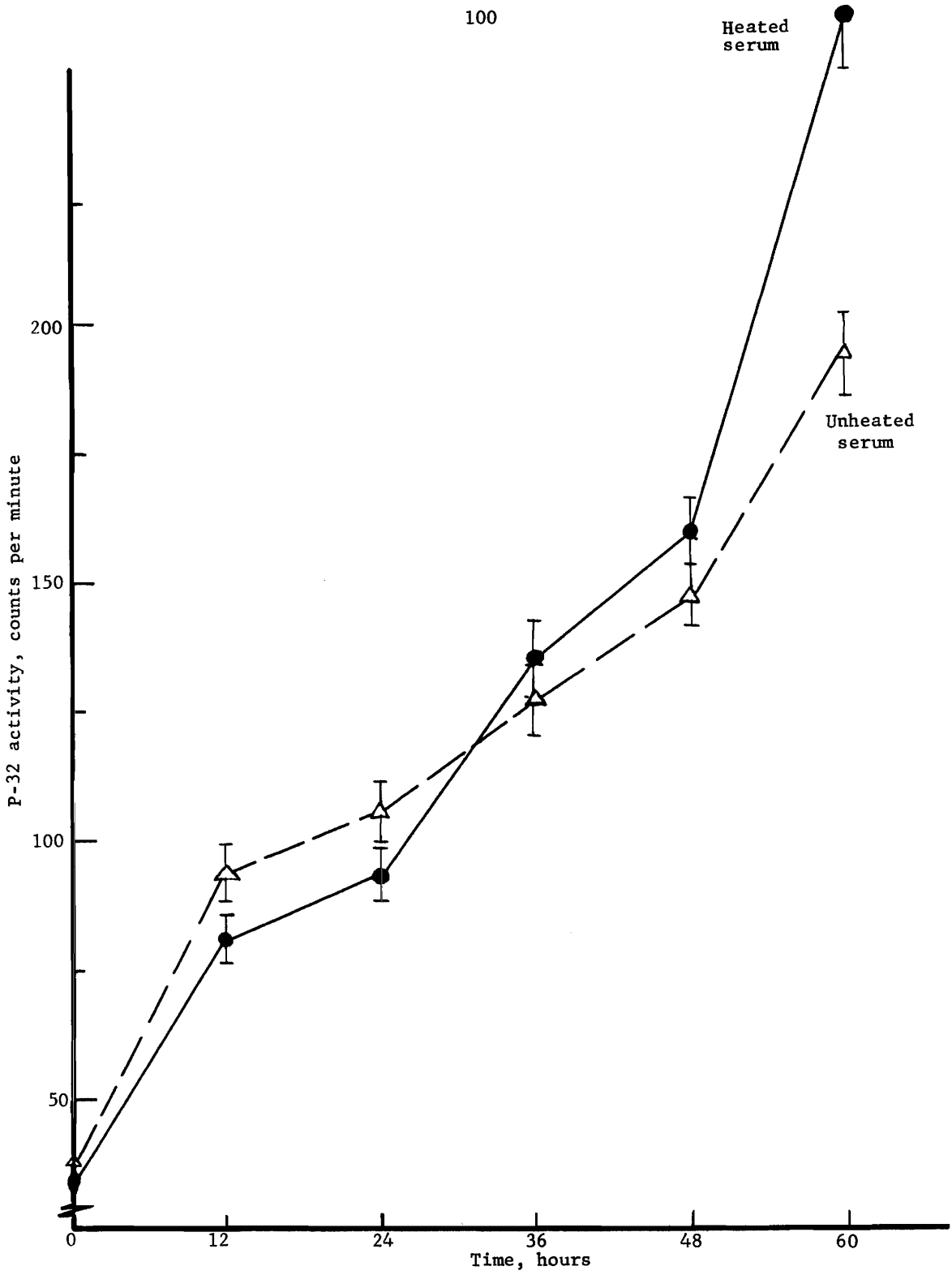

Figure 16. Relative rates of cytopepsis of H. capsulatum by "immune" macrophages in the presence of heated and unheated mouse serum.

Cytopeptic activity by normal cells (Figures 17 through 21) was also enhanced by the presence of heat labile factors, except again, in the presence of human serum (Figure 17). Furthermore, the transient increase was again noted with rabbit, calf and guinea pig serum (Figures 18, 19 and 20, respectively), while the increase with mouse serum (Figure 21) did not occur until 48 hours.

From these data it appears that heat labile factors do enhance digestion by both normal and "immune" cell populations, although this increase was not significant in the case of human serum. However, in this latter case, the P-32 released from cells suspended in menstruum containing the unheated serum was greater than after heating and gave the same transient response as with other sera.

3. Comparative effects of homologous and heterologous sera on digestion by normal and "immune" mouse macrophages. The final comparisons to be made from the data concern the effect of the various heated and unheated sera incorporated into the menstruums on rates of cytopepsis by normal and "immune" phagocytes. This was accomplished by plotting cpm of P-32 activity versus sampling time for (1) "immune" phagocytes in heated sera, (2) normal phagocytes in heated sera, (3) "immune" phagocytes in unheated sera, and (4) normal phagocytes in unheated sera. These results are given in Figures 22 through 25.

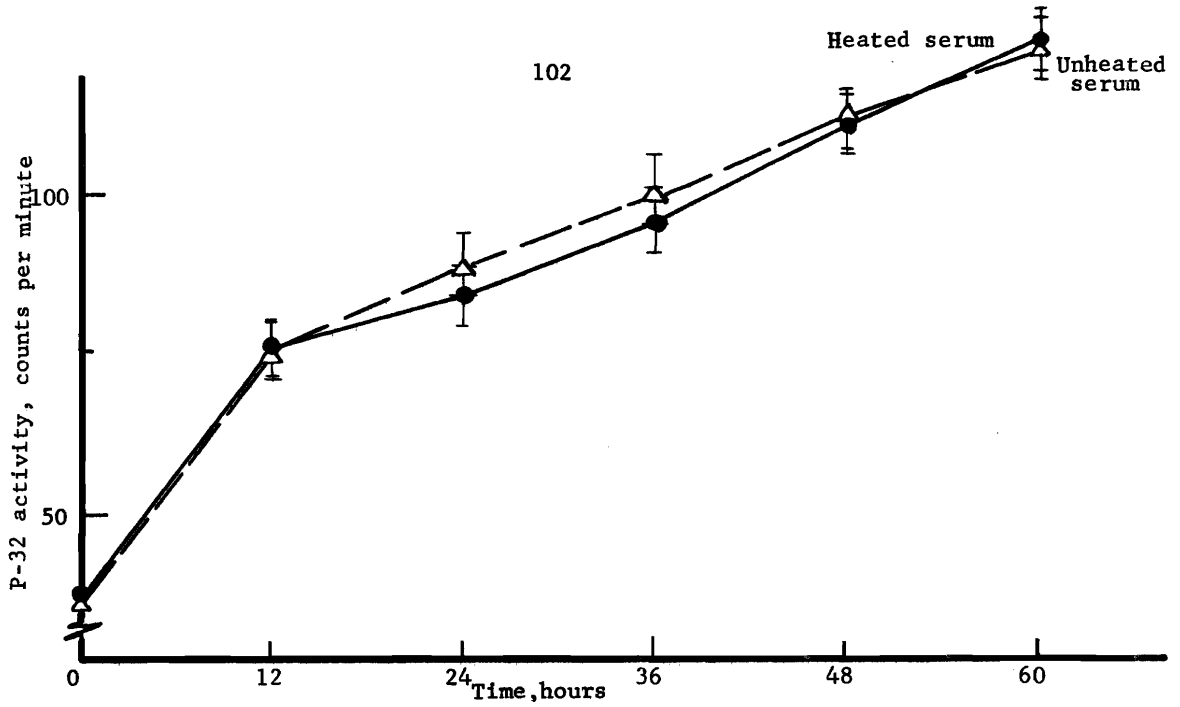

Figure 17. Relative rates of cytopepsis of H. capsulatum by normal macrophages in the presence of heated and unheated human serum.

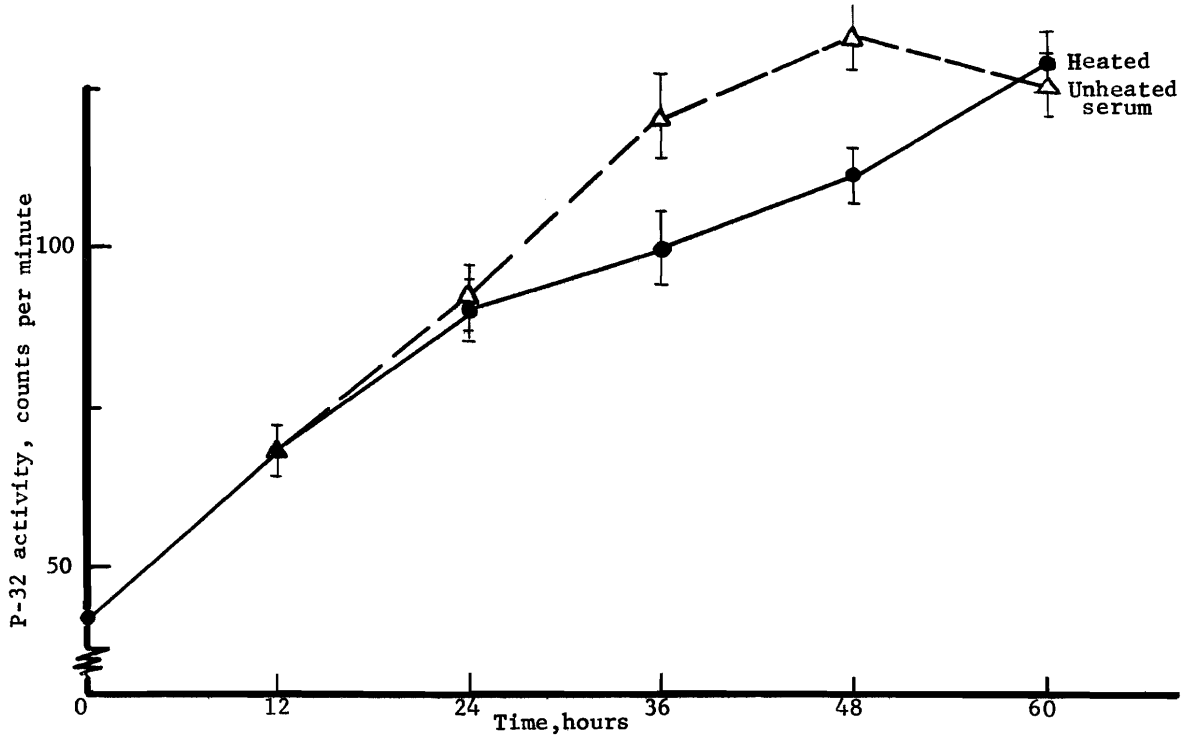

Figure 18. Relative rates of cytopepsis of H. capsulatum by normal macrophages in the presence of heated and unheated rabbit serum.

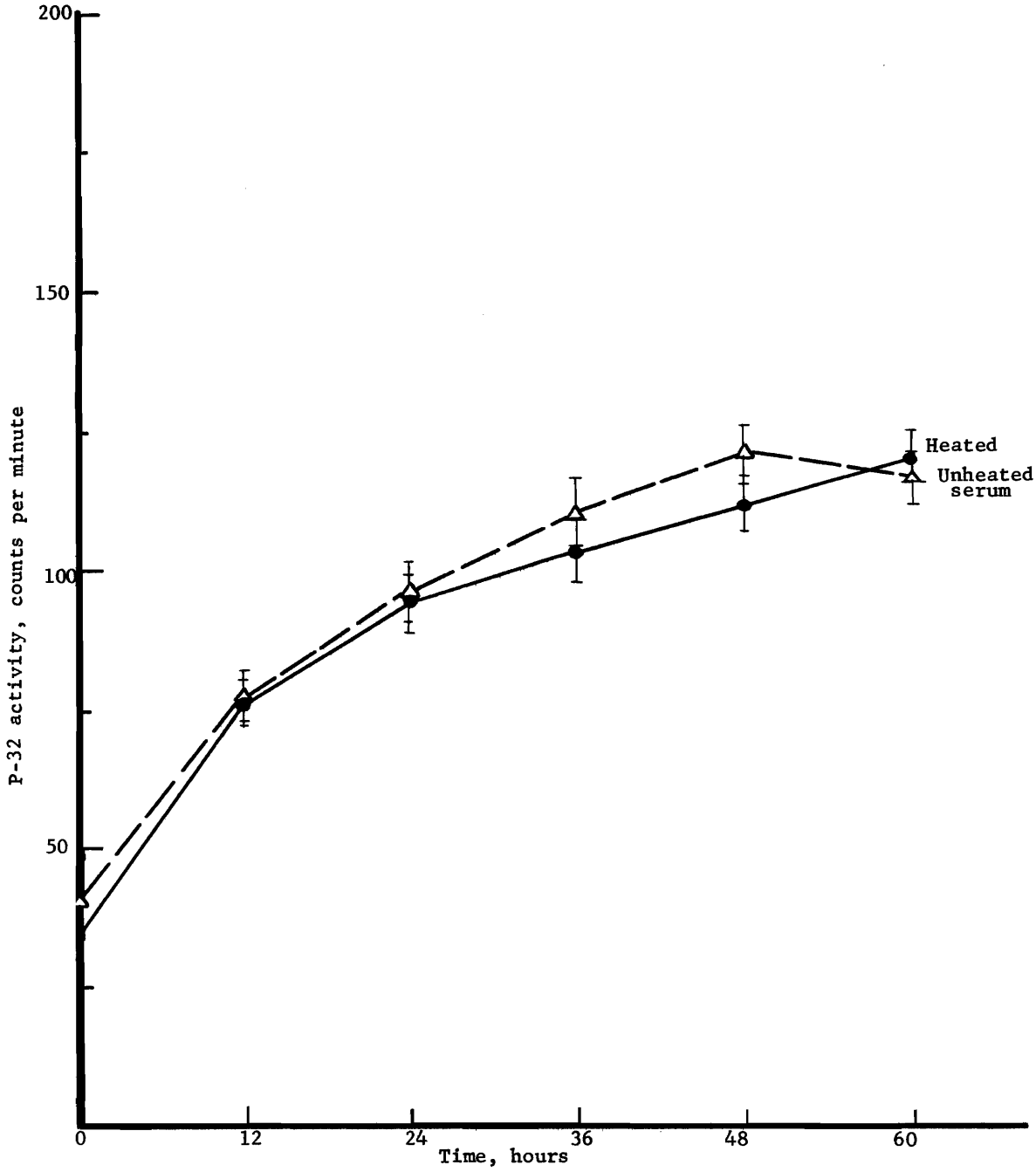

Figure 19. Relative rates of cytopepsis of H. capsulatum by normal macrophages in the presence of heated and unheated calf serum.

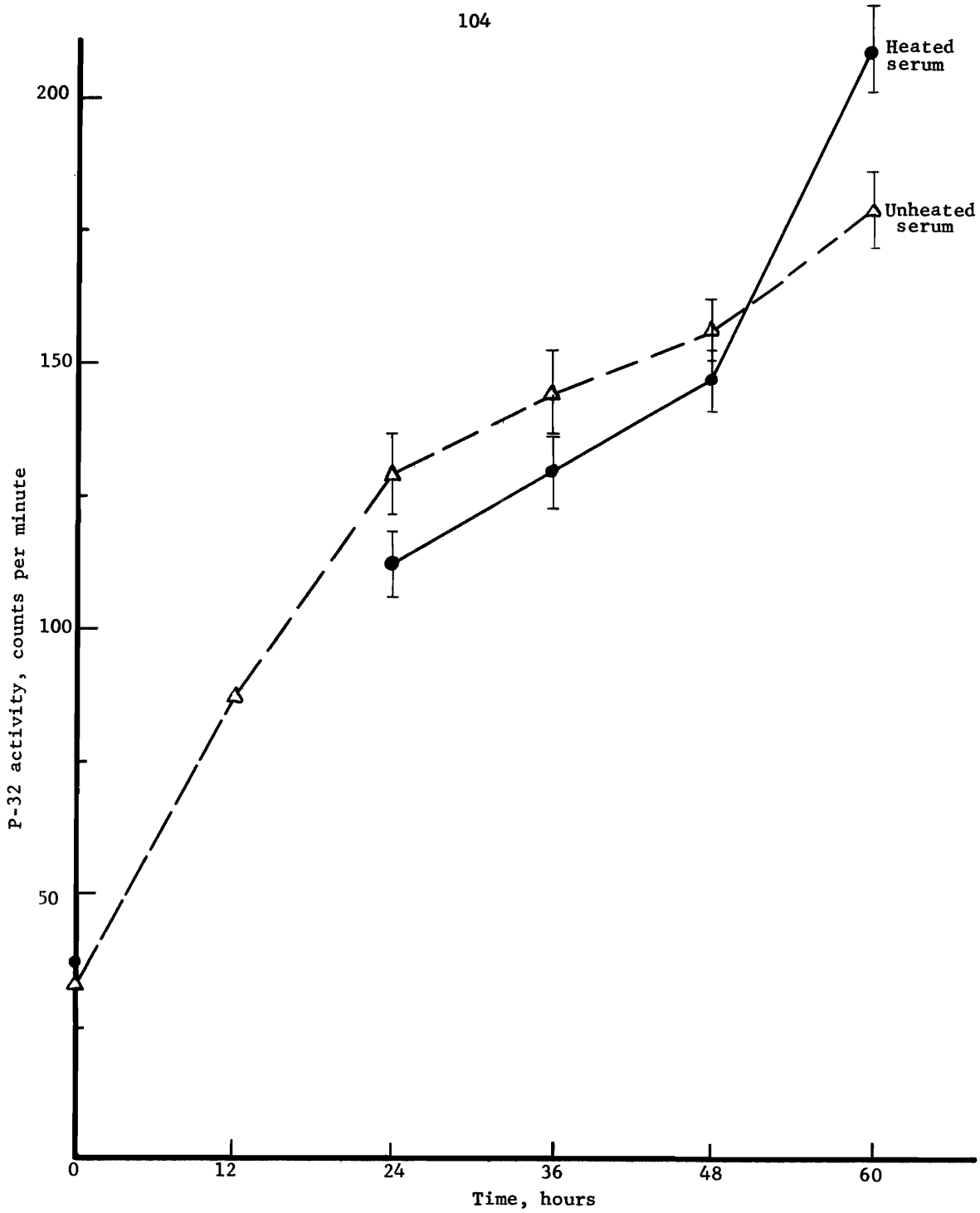

Figure 20. Relative rates of cytopepsis of H. capsulatum by normal macrophages in the presence of heated and unheated guinea pig serum.

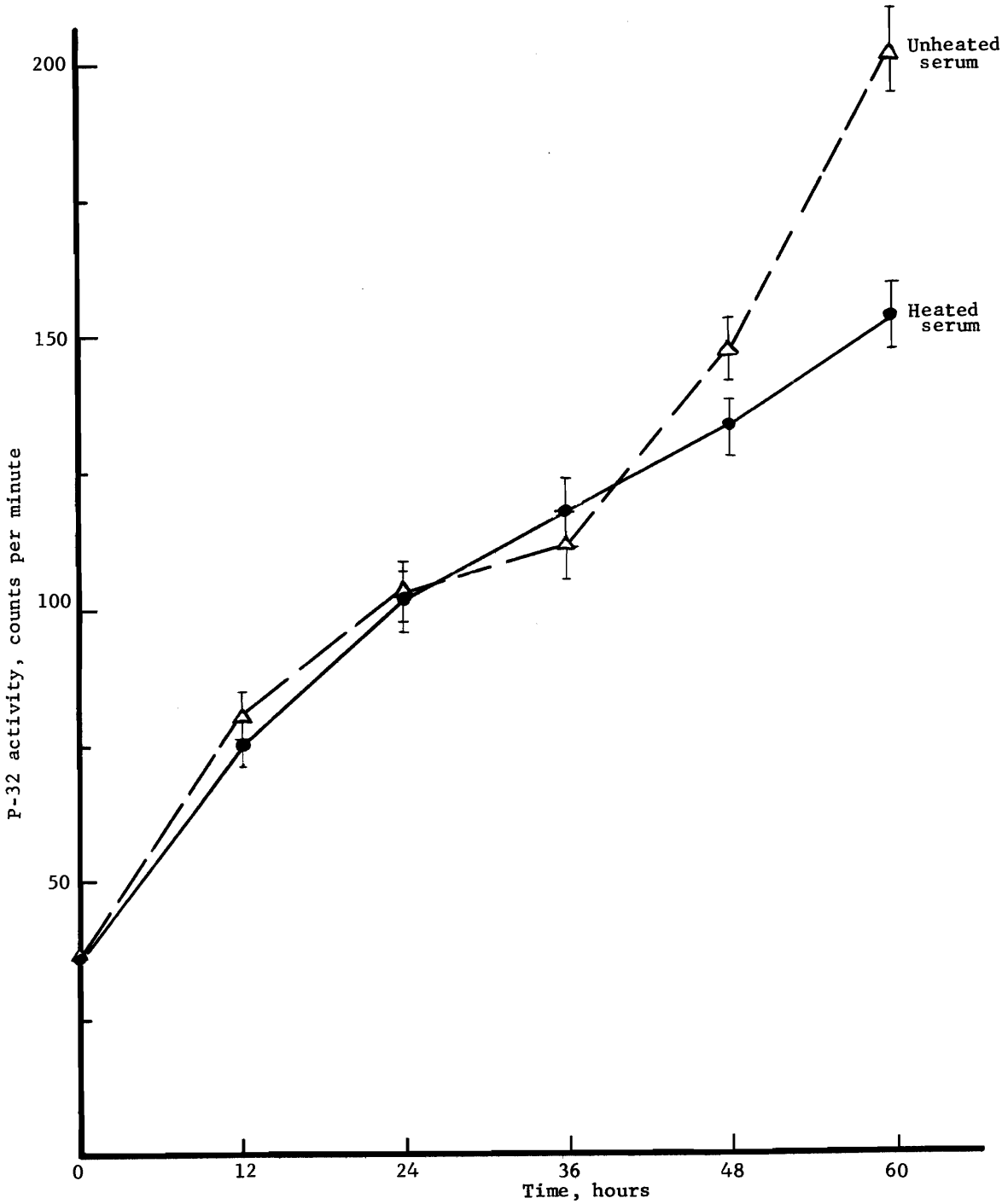

Figure 21. Relative rates of cytopepsis of H. capsulatum by normal macrophages in the presence of heated and unheated mouse serum.

Inspection of Figures 22 and 23, giving effects of heated sera on rates of cytopepsis by "immune" and normal phagocytes, show that mouse and guinea pig serum gave the highest rates of cytopepsis, being greater in the former case. Furthermore, the presence of human, calf and rabbit serum did not alter the cytopeptic rates by the "immune" cells or normal cells, these values being less in each serum than that obtained in guinea pig and mouse serum. It is interesting to note also that while mouse and guinea pig sera gave comparable results with "immune" cells (Figure 22) the latter caused more rapid digestion by normal cells (Figure 23).

The results shown in Figures 24 and 25, where "immune" and normal phagocyte activity in the presence of unheated sera are plotted, gave a picture similar to that observed with the cell populations in heated sera. However, it is obvious that the data were much more variable here, especially with "immune" cells (Figure 24). Generally speaking, guinea pig and mouse serum still brought about the higher rates of cytopepsis by both normal and "immune" cells, while human serum produced the least effect. Rabbit and calf serum promoted intermediate rates of digestion by the macrophages.

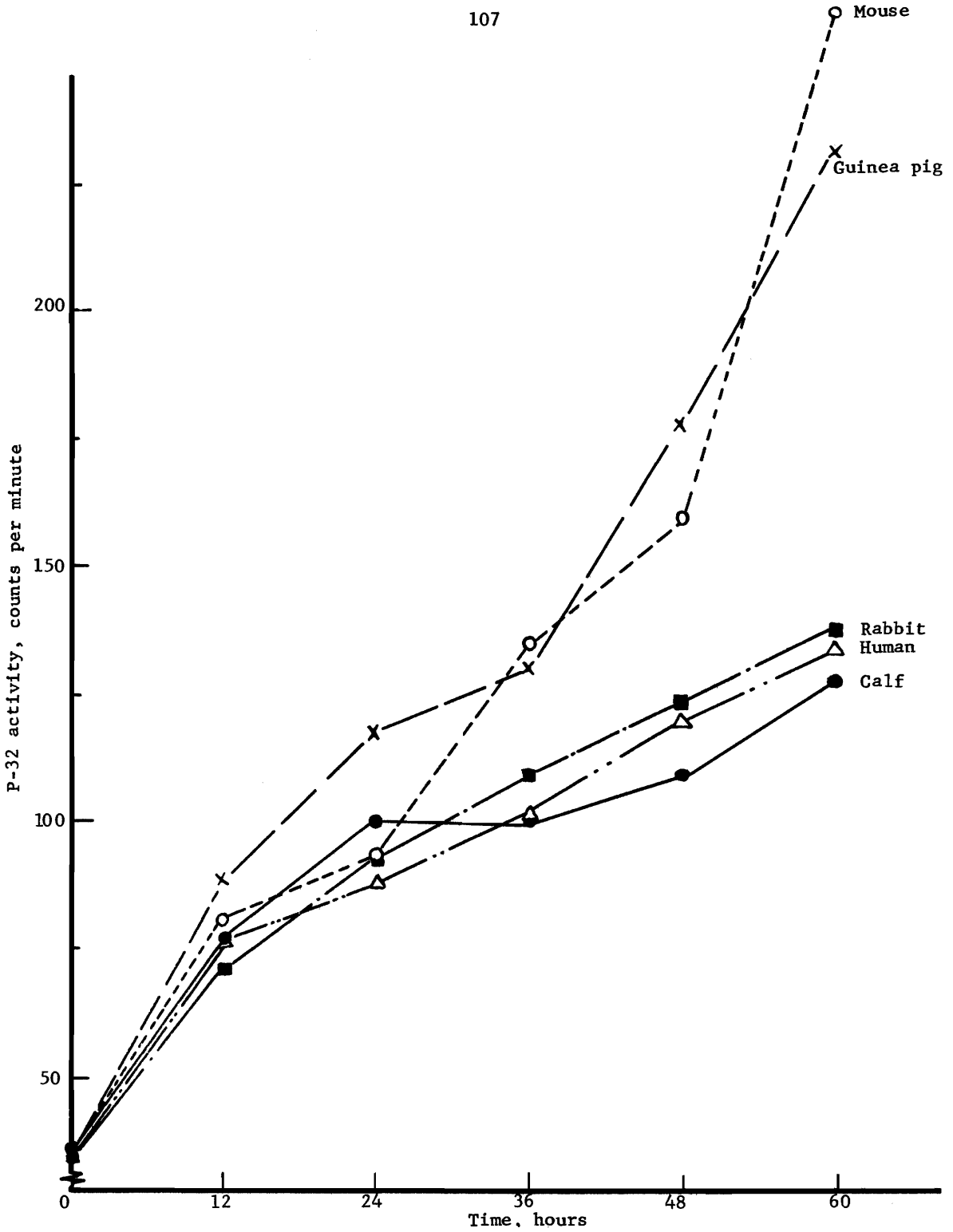

Figure 22. Comparative rates of digestion of H. capsulatum by "immune" macrophages in the presence of heated human, calf, rabbit, guinea pig and mouse serum.

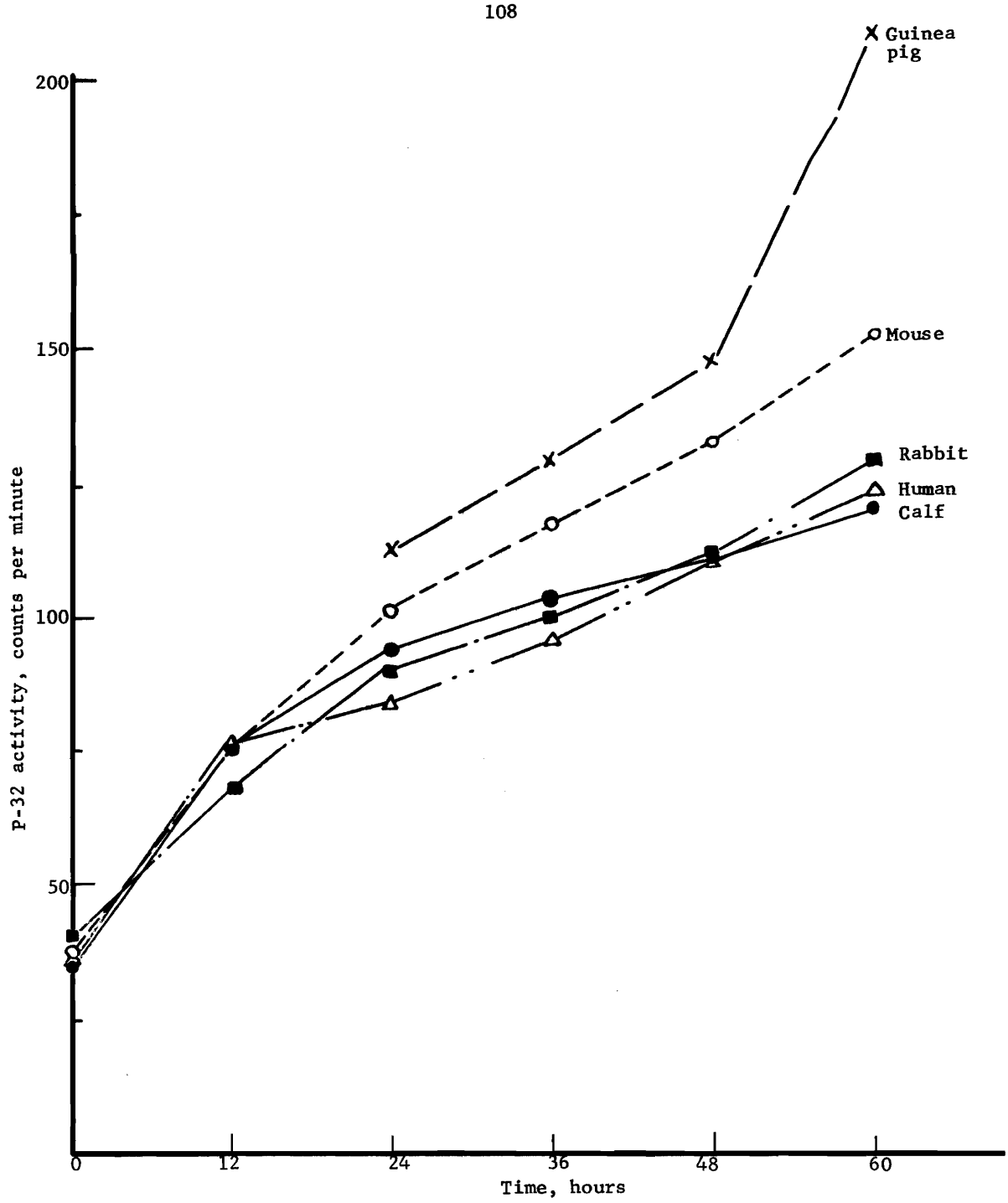

Figure 23. Comparative rates of digestion of H. capsulatum by normal macrophages in the presence of heated human, calf, rabbit, guinea pig and mouse serum.

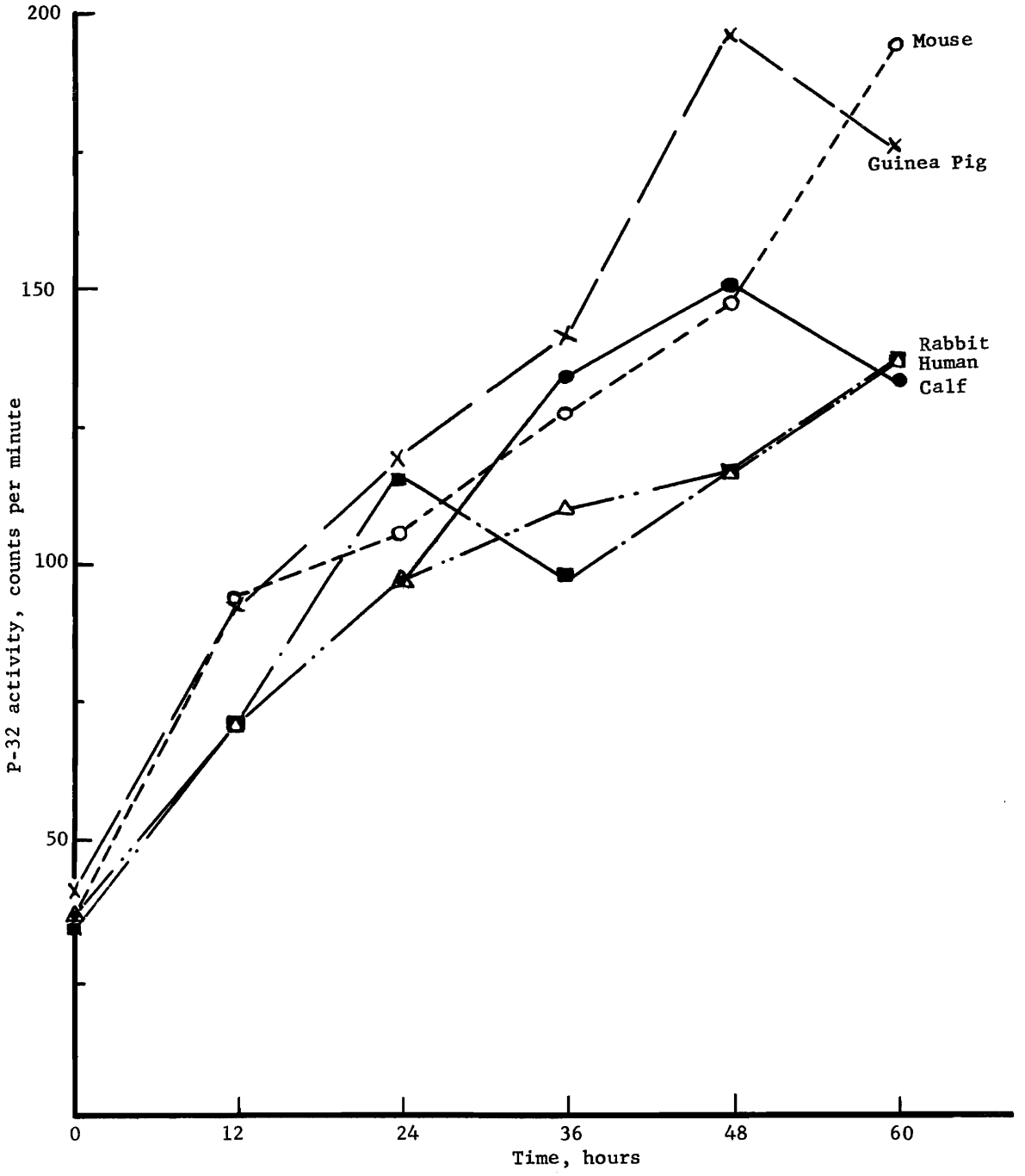

Figure 24. Comparative rates of digestion of H. capsulatum by "immune" macrophages in the presence of unheated human, calf, rabbit, guinea pig and mouse serum.

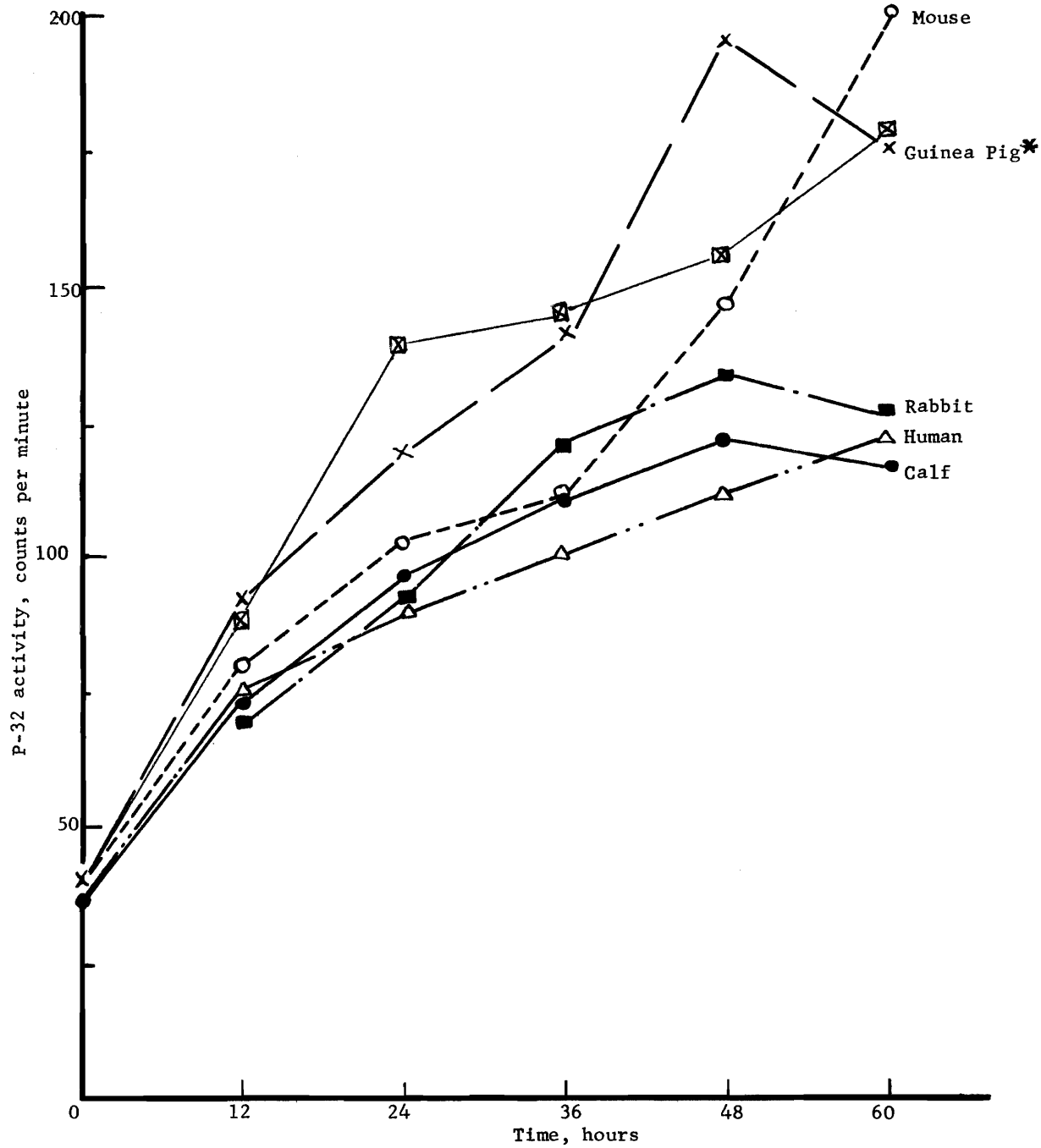

Figure 25. Comparative rates of digestion of H. capsulatum by normal macrophages in the presence of unheated human, calf, rabbit, guinea pig and mouse serum.

* ×——× is in error and should be deleted;

⊠——⊠ is the correct plot of data.

II. The Role of Complement and Antibody in Cellular Resistance

Several studies have been carried out in which differences in the activity of phagocytes from immune animals and those from non-immune animals have been distinguished. For example, Donaldson (1954) and Pomales-Lebrón and Stineburg (1957) reported that "immune" macrophages inhibited multiplication of Candida guilliermondi and Brucella abortus, respectively, over normal phagocytes in vitro. Also, Hill and Marcus (1960) found that "immune" macrophages from mice were distinctly more capable of carrying out cytopeptic activity in vitro with H. capsulatum as compared to normal phagocytes.

The studies referred to in the latter 2 papers were carried out in the absence of antibody; however, reports have been made which suggest that this humoral substance has little importance in cellular resistance after an organism has been ingested. Lurie (1942) was able to show that antibody did not enhance the bacteriostatic powers of "immune" phagocytes over normal phagocytes against the tubercle bacilli in vivo; Donaldson et al. (1956) demonstrated that the rate of cytopepsis of chicken red cells as well as of C. guilliermondi by mouse phagocytes was not increased by the presence of antibody; and Miya and Marcus (1961) found that the rate of digestion of H. capsulatum by normal and "immune" mouse macrophages was not altered by the presence of antibody against the organism.

The role of C' in phagocytosis and digestion by normal and "immune" phagocytes is less well known. Ward and Enders (1933), however, demonstrated that the presence of C' enhanced the phagocytosis of pneumococci

in the presence of antibody. Also, Moore (1919) reported that the opsonic index values of phagocytes from C' deficient guinea pigs were less than those from normal animals, suggesting that complement does aid in the ingestion process. Miya and Marcus (1961) have found a similar enhancing effect by the presence of heat labile substance(s) when the H. capsulatum-mouse macrophage system was employed. Furthermore, the enhancement was dependent on the amount of C' activity present up to an optimal level. These investigators also reported increased rates of digestion by the "immune" cells over normal cells in the presence of complement.

In the studies reported above, all data were comparative as to the phagocytic and digestive capacities of phagocytes from immune animals vs. nonimmune animals, while the effects of humoral substances, either alone or in combination with one another, within a population of "immune" cells or normal cells were not quantitatively shown. The purpose of this study has been to define the effects of complement and antibody on phagocytosis and digestion by "immune" and normal cell populations. Furthermore, comparisons between these two populations have been made with respect to their resistance to destruction by the infecting organism.

A. Effect of Complement Activity on Phagocytosis and Digestion.

Peritoneal exudate was collected from a group of normal mice, lightly centrifuged and the supernate discarded. The cells were washed once in CMF and resuspended in this solution. The phagocyte concentration was determined and adjusted to 1.25 million cells/ml with CMF. Twenty milliliters (total of 25 million cells) were added to each of six 50 ml glass stoppered flasks and 1 ml to each of several cover slip preparations.

After attachment had taken place the cell layer was washed once with CMF and 20 ml of the following solutions containing 2 x 10^5 yeast phase H. capsulatum per ml were added to the flasks: to the first flask was added cell maintenance fluid; to the second was added 70% Earle's BSS + 10% lactalbumin hydrolysate solution + 5 units/ml C' (3%) + 17% heated calf serum, that is, sufficient to give a total of 20% serum concentration; to flasks 3, 4, 5 and 6 the contents were similar to flask 2, except 10 units (6% guinea pig serum), 20 units (12% GPS), 30 units (18.1% GPS) per ml C', and C' heated at 56°C for 30 minutes (18.1% GPS) were added respectively, plus sufficient calf serum to give a total serum concentration of 20% in each flask. Also flasks were set up containing 10 ml of each of the above solutions with complement but without organisms or macrophages. These were used to determine the loss of C' activity with time on incubation at 35.5°C with shaking at slow speeds.

The original complement titer of the guinea pig serum was 166 units/ml as determined by the micro-Kolmer titration (Kolmer, Spaulding, and Robinson, 1951). The CMF was not anticomplementary.

Reference to Figure 26 shows the loss of C' activity with time at 35.5°C and shaking. It is apparent that under these circumstances complement activity decreased with time at a fairly constant rate. Therefore, flasks containing larger concentrations at the start of the experiment retained C' activity for the longest time.

Figure 27 shows plots of data from flask supernate and cover slip samples taken during the period of phagocytosis (4 hours) and digestion (4 hours). The data are plotted as per cent phagocytosis vs. time and

per cent digestion vs. time. The value for per cent phagocytosis was obtained as follows: (1) determine radioactivity (in counts per minute, cpm) given by samples taken at 0 time (start of experiment), 2 and 4 hours; (2) take the time of greatest difference in count from 0 time to equal maximum (100%) phagocytosis; (3) calculate as follows:

$$\% \text{ phagocytosis} = 100 \times \frac{\text{Count at 0 time minus selected time count}}{\text{Count at 0 time minus count at maximum phagocytosis}}$$

$$\text{or } \% P = 100. \quad \frac{(C_{T,0} - C_{T,1})}{(C_{T,0} - C_{T,max})}$$

Per cent digestion was based on radioactivity which occurred in the supernatant fluid. In these experiments, digestion was assumed to occur following the 4 hour ingestion period. Maximum possible cytopepsis was assumed to be numerically represented by the difference in cpm from 0 time to 4 hours after the start of the experiment. The value for per cent digestion was obtained as follows:

$$\% \text{ cytopepsis} = 100 \times \frac{\text{Count at selected time minus count at 0 time cytopepsis}}{\text{Theoretical maximum cytopepsis}}$$

$$\text{or } \% C = 100. \quad \frac{(C_{T,a} - C_{T,0'})}{C \text{ max cy}}$$

Examination of Table 6, which is an excerpt of data from this experiment, will aid in making the methods of calculation more clear.

The per cent relative activity of cover slip preparations was merely the cpm at zero time of digestion minus cpm for any particular time observed multiplied by 100. The plot of these data represent the actual activity retained within the macrophages due to ingested P-32

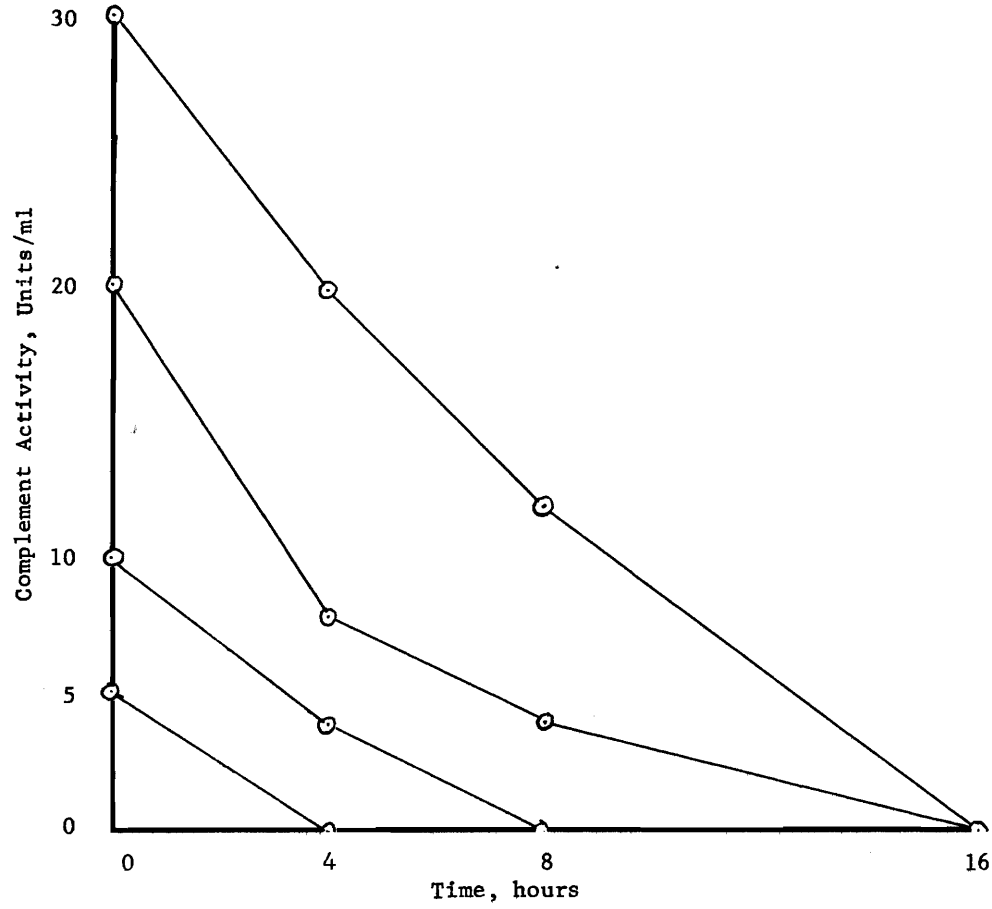

Figure 26. Loss of complement activity with time; C' diluted in 70% BSS + 10% LAH solution and sufficient heated calf serum to give 20% total serum concentration, then incubated at 35.5°C with shaking.

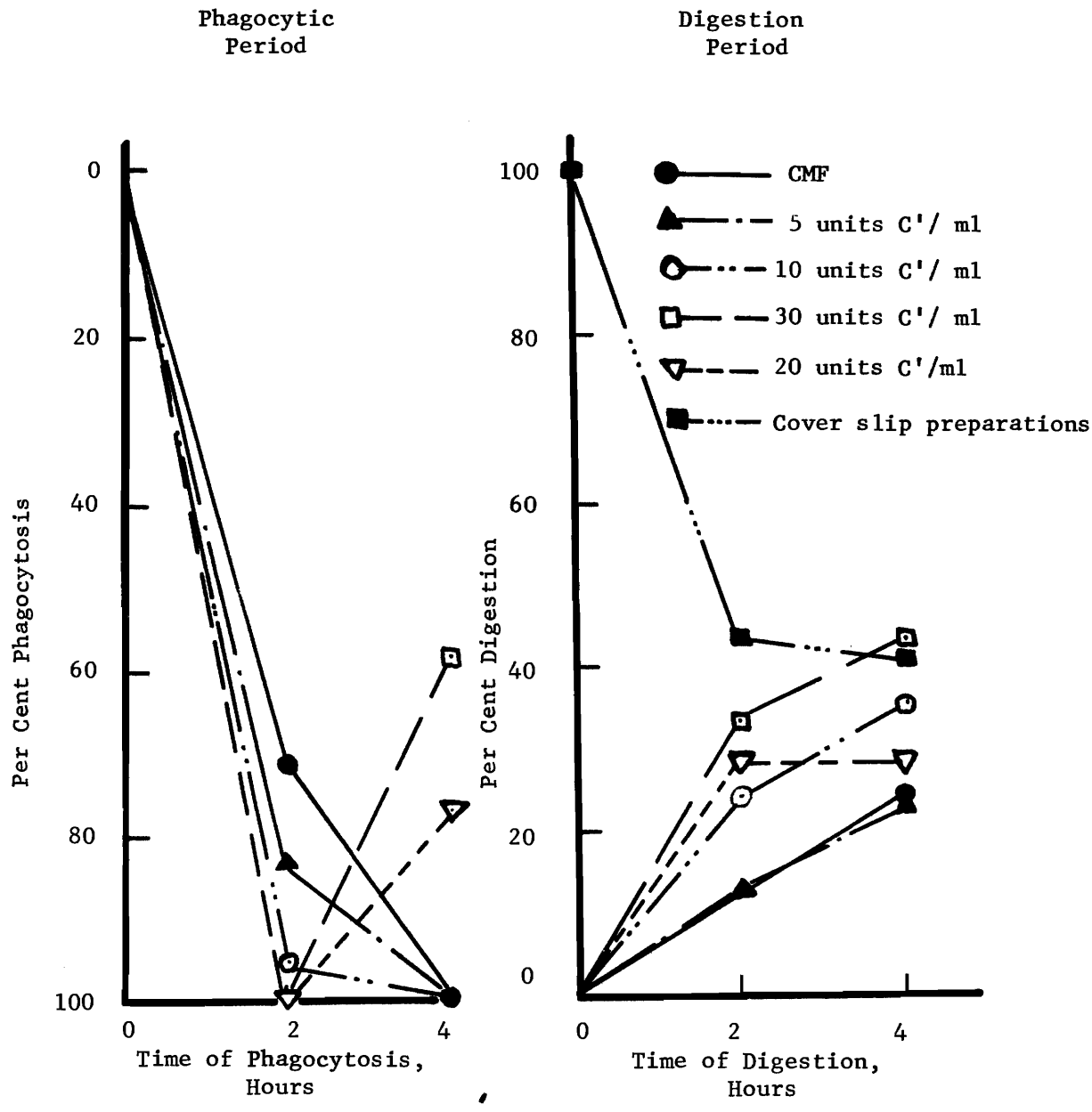

Figure 27. Effect of varying guinea pig complement concentrations on phagocytosis and digestion by normal mouse macrophages.

TABLE 6

Excerpt of data showing per cent phagocytosis and cytopepsis of P-32 labeled H. capsulatum by normal mouse phagocytes.

Sample period	Substance being tested	Time of sample (hours)	cpm	Difference in cpm from 0 time	Per cent
Phago-cytosis	Heated C'	0	431	0	0
		2	262	169	78.6*
		4	216	215	100.0
Diges-tion		0	33	0	0
		2	48	15	7.0
		4	76	43	20.0+

* % phagocytosis at 2 hours; $\% P = (100) \frac{(431 - 262)}{(431 - 216)} = 78.6$

\+ % cytopepsis at 4 hours; $\% C = (100) \frac{(76 - 33)}{(431 - 216)} = 20.0$

labeled yeast phase organisms. Therefore, reduction of activity suggests reduction in the number of intact organisms presumably due to death and intracellular digestion.

Figure 27 shows the overall effects of C' on phagocytosis and digestion. It appears that the rate of phagocytosis was altered by the C' activity present although this was not found in later experiments. Thirty units/ml, the maximum concentration used, gave the most rapid uptake,

while samples from flasks containing no complement showed the slowest uptake of H. capsulatum.

It was noted that in 2 flasks, namely those containing 20 and 30 units/ml C', the per cent radioactivity was less after 4 hours phagocytosis than that at the end of 2 hours. This was attributed to increased digestion in these two flasks. Hence, at the end of 2 hours, maximum ingestion had taken place (i.e. the largest difference in cpm of the supernate compared to 0 time), and digestion had overtaken ingestion, thereby causing the supernate activity to rise.

This reasoning is borne out by the plot of data for the digestion period seen in Figure 27. Again, the macrophages in the presence of the higher concentration of C' gave the most rapid rate of digestion.

The relative radioactivity of cover slip preparation, which contained labeled organisms and macrophages in CMF, is plotted in Figure 27 and shows an inverse relation to activity appearing in the supernate fluid samples. However, these values were not correlated with the supernate samples from the flask containing CMF, possibly because of the differences in conditions present between them, such as fluid volume, which gives rise to differences of the amount of agitation of the contents on the shaker and eventual pH differences on incubation.

It is apparent, however, that under the experimental circumstances 30 units/ml C' gave maximum phagocytosis and digestion. For this reason, this concentration was used in the following experiment which compares the effects of antibody and complement on phagocytosis and digestion.

B. Effects of Complement and Antibody on Phagocytosis and Digestion.

The purpose of this experiment was to determine the effects of C' and antibody on phagocytosis and digestion within a single population of cells ("immune" or normal macrophages) and between two different populations of cells ("immune" vs. normal macrophages).

Peritoneal exudates were collected from normal mice and mice immunized with H. capsulatum. These were washed and adjusted to a constant number with CMF as previously described. Twenty million phagocytes were then added to 50 ml glass stoppered flasks and 1.5 million to each cover slip preparation. One set of 5 flasks contained 20 million "immune" cells/flask and another set contained 20 million normal cells/flask. Similarly, one set of cover slip preparations contained 1.5 million "immune" cells per preparation and another set contained 1.5 million normal cells per preparation.

After overnight incubation, during which the macrophages attached to the glass, the supernatant fluids were aspirated out and the attached cells washed once in CMF.

Antibody was introduced by pretreating P-32 labeled H. capsulatum with specific rabbit antiserum (complement-fixing titer of 1:256) at 37°C for 0.5 hour with frequent shaking. After this the antibody-treated organisms were washed 3 times with Earle's BSS and resuspended in CMF. As a direct control for the antibody treatment of H. capsulatum, an equal number of P-32 labeled organisms was treated in a like manner with normal rabbit serum (NRS), washed and resuspended in CMF.

The concentration of untreated yeast phase organisms, antibody treated cells, and NRS treated cells was determined by direct counting and then adjusted to equal numbers with CMF or a medium consisting of 30 units/ml C' (20% by volume), 10% lactalbumin hydrolysate solution, and 70% Earle's BSS. Next, addition of menstruums and organisms was made to the flasks and cover slips containing macrophages, as outlined in Table 7.

TABLE 7

Flask and cover slip contents for determination of the effect of C' and antibody on phagocytosis and digestion by normal and "immune" macrophages.

Flask or Cover slip	Menstruum and Amount	H. capsulatum 1×10^6 cells/ml
1 N* & 1 I* flask	CMF, 20 ml	Untreated
2 N & 2 I flask	Earle's BSS + LAH + C'**, 20 ml	Antibody treated***
3 N & 3 I flask	" " ", 20 ml	Normal rabbit serum treated***
4 N & 4 I flask	CMF, 20 ml	Antibody treated
5 N & 5 I flask	" , 20 ml	NRS treated
N & I cover slip	CMF, 1.5 ml	Untreated

* N indicates normal phagocyte-containing flask, I indicates "immune" phagocyte-containing flask.

** C': 30 units/ml at 0 time.

*** Treated for 30 minutes at 37°C with frequent shaking/and washed 3 times with BSS.

Immediately after addition of the cell suspensions to their respective flasks (Table 7) supernate samples were taken for radioassay, as were cover slip preparations.

Phagocytosis was allowed to take place for 3 hours at 35.5°C with continuous slow shaking. Samples were taken at the end of 2 and 3 hours. After the third hour, the supernatant fluids were removed and the macrophages containing the ingested H. capsulatum organisms were washed once in CMF. Fluids composed of the same constituents previously contained in each flask or cover slip preparation were added back and the digestion period followed for 32 hours, while incubation continued at 35.5°C without agitation except before sampling. Figures 28 and 29 show the results of this experiment.

First, comparing the results within a population of cells, Figure 28 shows the effects of complement and antibody on phagocytosis and digestion by "immune" cells. It was noted that in this particular experiment, neither antibody nor C' enhanced phagocytosis. This was not in agreement with the previously described experiment where C' appeared to alter the phagocytic processes. This discrepancy in results is possibly explained by the excessive number of infecting organisms added to each flask and cover slip preparation as compared to the previous experiment. Here, 1×10^6 yeast phase organisms/ml were employed, whereas previously only 2×10^5 organisms/ml were used. This extra amount of cells, it may be suggested, gave maximum contact between the phagocytes and the infecting organism causing maximum ingestion to take place without respect to the humoral factors present. In this fashion, masking may have occurred in

the presence of any enhancing effect that could have been brought about by these substances.

The portion of Figure 28 showing the effects of humoral substances on "immune" cell digestion indicates that C' had the greatest single enhancing effect. The effect was much more than that due to antibody alone, which did, however, bring about an increased rate of digestion as compared to the control. The combination of antibody and C' provided the conditions for optimal digestion, rates being more rapid under these circumstances than under any other condition employed. Therefore, from these data, it appears that antibody and complement both may play a role in digestion by "immune" cells.

In Figure 29, which shows plots of data on supernate fluid and cover slip data from the normal cell cultures, similar overall results, except in the CMF containing flask, were obtained. However, it was noted that the per cent digestion increased much more rapidly than did the rates exhibited by "immune" cells. This is accounted for not by increased rates of digestion by these cells, but by the macrophages being destroyed by the overwhelming doses of organisms. That is, the normal phagocytes literally brought about their own destruction by over-ingestion of H. capsulatum, with the result that non-digested organisms appeared back in the supernatant fluid causing an increased amount of activity in supernate samples taken during this period. This was apparent both from gross observation of the flasks during incubation, when clumps of macrophages could be seen in the medium during the early digestion phase and by microscopic examination of the cover slip samples taken during various

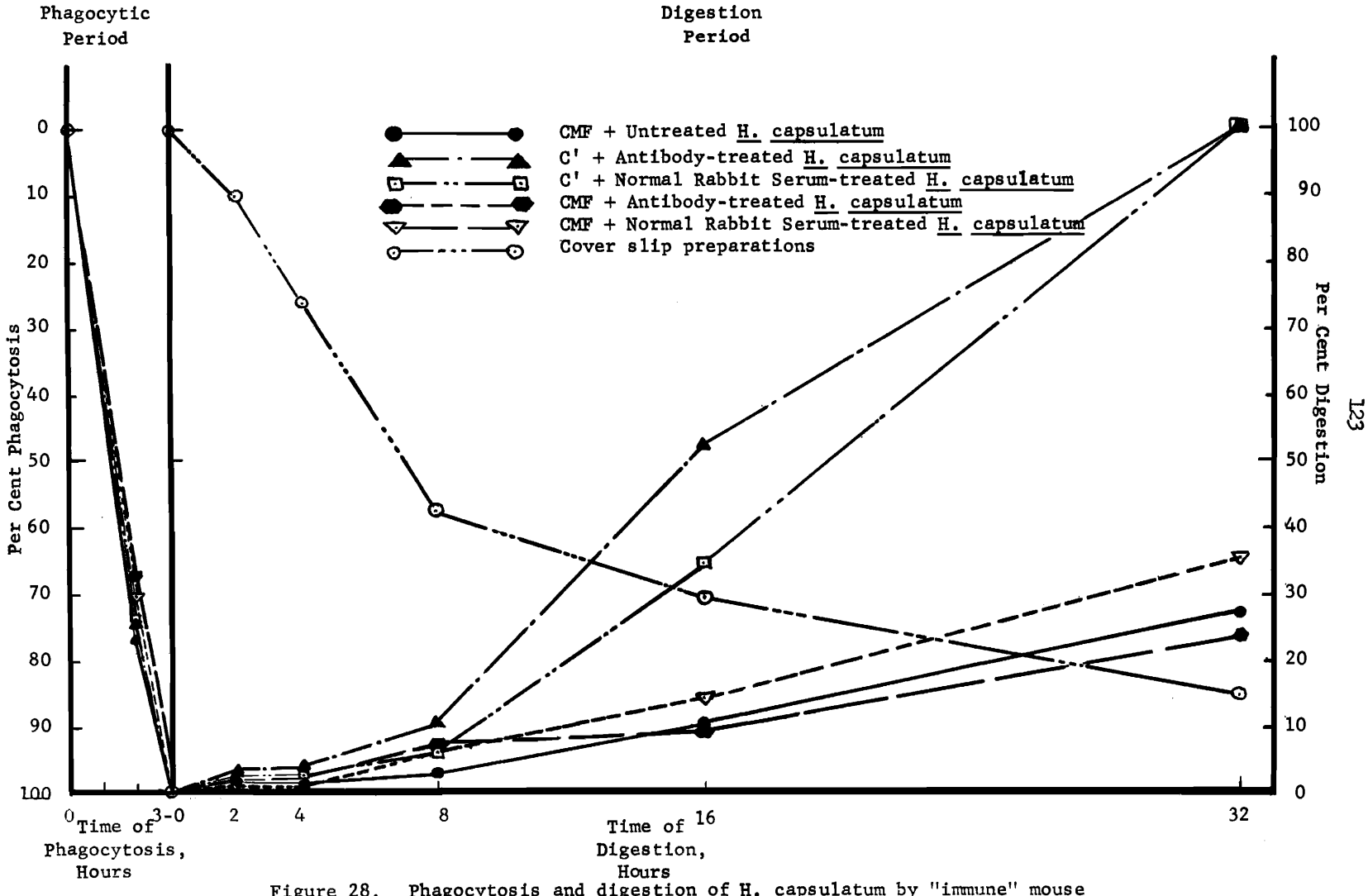

Figure 28. Phagocytosis and digestion of H. capsulatum by "immune" mouse macrophages in the presence and absence of C' and antibody.

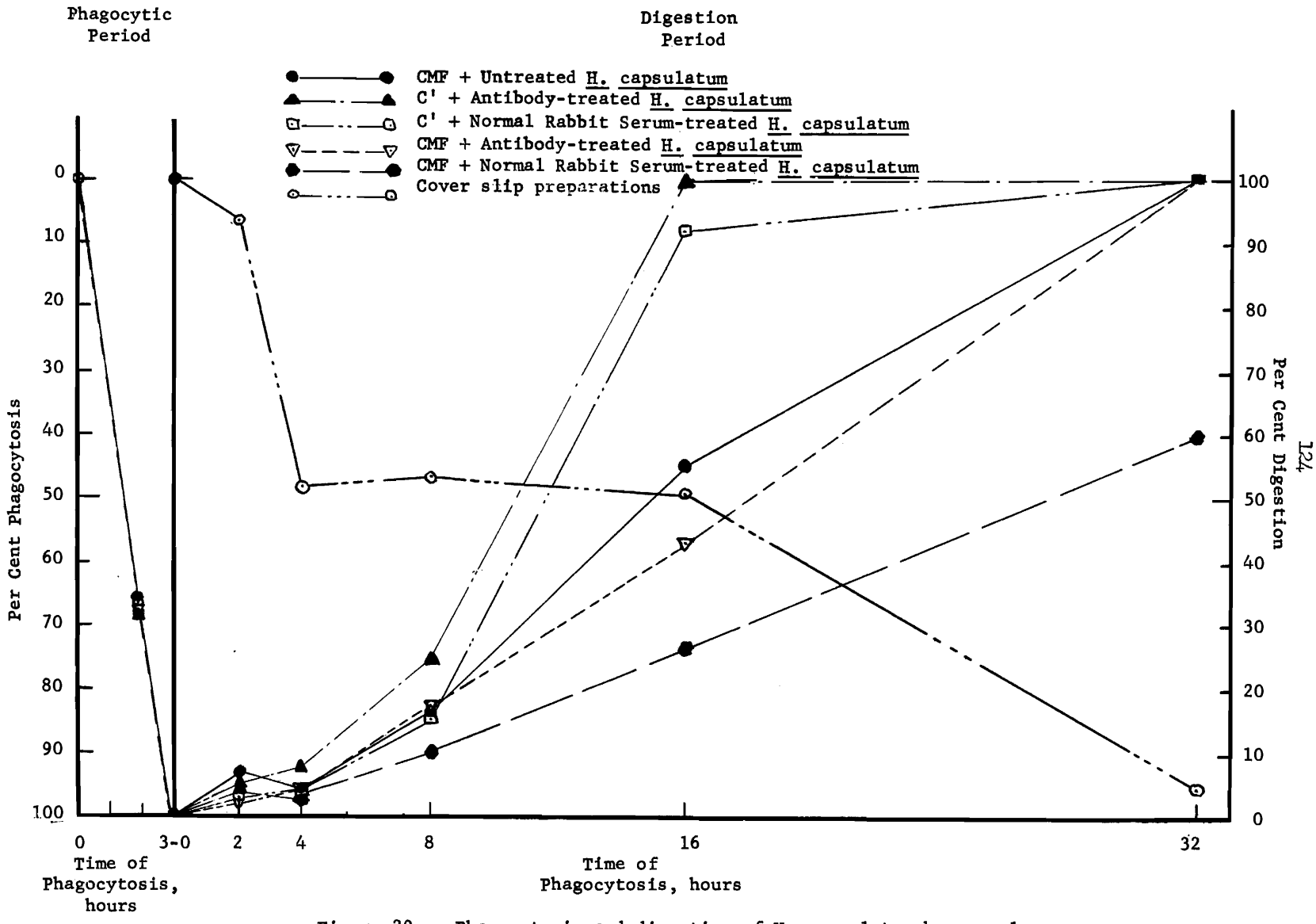

Figure 29. Phagocytosis and digestion of H. capsulatum by normal mouse macrophages in the presence and absence of C' and antibody.

time intervals. These observations established the presence of damaged, fragmented normal macrophages and disattachment of them with passage of time. The latter fact was determined by making direct counts of the number of macrophages present in 10 randomly selected microscopic fields at 0 time phagocytosis, and comparing this with the number present at other times during the experiment (Table 8).

TABLE 8

Per cent cell destruction caused by H. capsulatum.

Period of Sampling	Sample Time, hr.	Per cent loss of cells*	
		"Immune"	Normal
Phagocytosis	0	0	0
	2	0	0
	3	1	14
Digestion	0	0	14
	2	0	40
	4	16	71
	8	13	76
	16	14	85
	32	38	89

* Obtained from 10 randomly select microscopic field per cover slip preparation

The "immune" cells were not subject to the destruction due to ingestion of large numbers of yeast phase organisms to such a large extent

as normal cells as shown in Table 8. Although some cells in this group were lost from the glass, the major portion maintained themselves until the sixteenth hour, then, between the sixteenth and thirty-second hour, approximately 25% of the population was lost, probably due to the intracellular multiplication of H. capsulatum.

The fact that some cell loss occurred in the "immune" cell population and many from the normal cell population does not invalidate the data of Figures 28 and 29 for comparisons within each of the cell populations, since it can be assumed that because the same incubation temperature, methods of incubation, same numbers of macrophages, and same numbers of infecting organisms were present per flask, the same numbers of macrophages were lost per unit time. Under these circumstances the data may still be treated as valid for comparison. This analysis is borne out by the comparable gross results obtained within the two cell populations.

It should be noted also that the degree of phagocytosis by both "immune" and normal cells was nearly the same. This is shown both by the supernatant activity during phagocytosis and by gross microscopic observations carried out on the cover slips.

The radioassay of cover slips, as with the previous experiment, gave an inverse relation between counts given here to that obtained with the supernate samples. However, no direct correlation can be obtained in terms of cpm.

Comparisons between the populations of cells by the radioassay method is not considered valid here, because of the decrease in viable macrophages in the normal group of cells as compared to the "immune" group.

However, comparisons between "immune" and normal cell digestion could be made by microscopic examination of macrophages remaining on the cover slips for the degree of digestion having taken place. This could be distinguished by the presence of Schiff positive material in these cells. Therefore, 100 macrophages per cover slip were examined for each time interval and the results obtained are given in Table 9.

TABLE 9

Per cent digestion by normal and "immune" cells in CMF as determined by microscopic examination.

Period of Sampling	Sample Time, hr.	Per cent Digestion	
		"Immune"	Normal
Phagocytosis	0	0	0
	2	0	0
	3	0	0
Digestion	0	0	0
	2	4	5
	4	6	6
	8	13	6
	16	32	27
	32	60	26

It can be seen from this Table that the "immune" cells were capable of digesting H. capsulatum at a more rapid rate than normal cells, even though maintained in CMF in the absence of labile humoral components and

antibody. However, data given here for "immune" cells did not correlate well with the per cent digestion by "immune" cells in flasks, the latter being comparatively low during the latter stages of the experiment. This may possibly have been due to a time lag between the presence of Schiff positive material in the macrophage and the excretion of digested material back into the supernatant fluids. If this were the case, the difference in results between the 2 methods of measurement would be expected.

A comparison cannot be made between the data of Table 9 for normal cells and that given in Figure 28 because of the cell destruction by intracellular infection being included in the data in the latter case.

III. The Role of Complement and Complement Fractions in Phagocytosis and Cytopepsis.

Prior data appears to implicate heat labile factors of normal sera in enhancing cytopepsis of H. capsulatum by normal and "immune" mouse macrophages. To further investigate the possibility that complement or one of its components might be the major factor involved, the following experiments were undertaken.

The procedure employed in this investigation was essentially that described under "Materials and Methods," using "Method I" for cell culture. That is, peritoneal exudates were obtained from a group of normal mice, lightly centrifuged, then washed three times in cell maintenance fluid (CMF). The macrophages were resuspended in CMF, counted in a hemocytometer and then adjusted to a concentration of 1.25 million cells per ml. Twenty milliliters (containing 25 million cells) of this

suspension were added to each of five 50 ml glass stoppered flasks and then incubated at 35.5°C for 48 hours without agitation.

After incubation, during which time the phagocytes attached to glass, the supernatant fluids were removed by aspiration, the attached cells washed twice with CMF, and once with Earle's BSS. Next, suspensions containing 1 x 10^5 P-32 labeled H. capsulatum per milliliter were added, as outlined in Table 10. It should be noted that a 24 hour culture of P-32 labeled yeast phase H. capsulatum was employed here rather than the three day culture previously used. Also, the cells of this culture were allowed to respire 4 hours rather than overnight as done in other experiments.

Actual complement activity of the solution in Flask 1 after dilution in incomplete CMF, but before addition of organisms, was 125 units/ml. Computed levels of component activity of the various C' reagents after dilution in incomplete CMF are given in Table 11. It can be seen that R4 actually lacks C'2 as well as C'4, thereby giving this reagent the same composition as midpiece, but having higher component titers.

Immediately following addition of the H. capsulatum suspensions to their respective flasks (Table 10) supernatant samples were obtained for radioassay. Phagocytosis was allowed to proceed for 4 hours with slow shaking at 35.5°C with samples being again taken at the end of this period.

After this, the supernatant fluids were again removed from all flasks, and the attached macrophages with their ingested P-32 labeled organisms washed 3 times with Earle's BSS. The same menstruum previously

TABLE 10

Flask contents for determination of the effect of complement and complement reagents on phagocytosis by normal mouse macrophages

Flask	Menstruum
1	80% Incomplete CMF* + 20% whole C'
2	60% Incomplete CMF + 40% midpiece**
3	" " " + 40% endpiece**
4	80% Incomplete CMF + 20% R3
5	" " " + 20% R4

* Incomplete cell maintenance fluid consisting of varying amounts of Earle's BSS and 10% lactalbumin hydrolysate solution.

** M and E diluted 1:2 in preparation.

TABLE 11

Complement and complement reagent hemolytic activity after dilution in incomplete cell maintenance fluid

Flask	Complement Fraction	C' Titer units/ml	Component Titers units/ml*			
			C'1	C'2	C'3	C'4
1	Whole C'	125	Not determined			
2	Midpiece	-	80	<4	40	<4
3	Endpiece	-	0	20	125	320
4	R3	-	160	125	0	640
5	R4	-	320	<4	320	<4

* Computed values based on titers obtained prior to dilution (see Table 1).

contained in each flask was then readded without organisms and the digestion period followed for 60 hours at 35.5°C without agitation except prior to taking supernatant samples. These samples were centrifuged for 20 minutes at 25,000 x g, then 1 ml of the cell-free supernatant fluid was placed in planchets, dried and radioassayed. Plate counts on the supernatant fluids were not carried out in this experiment, although occasional samples from the centrifuged fluids were taken to insure the absence of free P-32 labeled H. capsulatum. Table 12 and 13 show the results of this experiment.

A. Ingestion Phase.

Table 12 gives the relative rates of ingestion of H. capsulatum by normal mouse macrophages as determined by P-32 activity, both in counts per minute (cpm) and per cent, at the end of 4 hours. Cpm represents the difference in radioactivity found at 0 time and that at the end of 4 hours, and therefore, the relative numbers of H. capsulatum taken up by the phagocytes.

It can be seen that rates of ingestion in the presence of whole C' or C' reagents did not appear to be significantly different from each other with the exception of the menstruum containing R3. However, the increased ingestion in the presence of R3 was probably due to a higher number of organisms originally present in the flask than those containing whole C' or other reagents. Therefore, this result is not considered to be a difference attributable to the humoral factor.

B. Digestion Phase.

Table 13 outlines the results obtained during the period of cytopepsis.

TABLE 12

Relative ingestion rates of P-32 labeled H. capsulatum by normal mouse macrophages in the presence of C' or C' reagents

Flask	C' Fraction	Ingestion at End of 4 Hours	
		cpm*	per cent
1	Whole C'	260	100
2	Midpiece	201	100
3	Endpiece	217	100
4	R3	407	100
5	R4	297	100

* cpm computed by subtracting cpm at 4 hours from cpm at 0 time.

TABLE 13

Relative rates of digestion of H. capsulatum by normal mouse macrophages in the presence of C' or C' reagents

Sample Time, Hours	Per Cent Digestion				
	Whole C'	Midpiece	Endpiece	R3	R4
0	0	0	0	0	0
4	0	0	1.0	0.1	0.2
12	1.0	2.5	2.0	1.5	1.4
24	-	3.6	5.8	2.4	-
36	4.4	7.4	7.2	5.2	6.8
48	5.6	8.4	10.2	4.0	8.5
60	8.5	13.1	14.7	12.4	12.8

These results are expressed as per cent digestion of the ingested organism with time. From these data it appears that the lack of one or more C' components from this system does not significantly alter the rates of cytopepsis, with the degree of digestion ranging from 8.5 to 15% at the end of 60 hours. It is interesting to note that in the flasks containing whole C' and R3 cytopeptic rates were slightly retarded. However, the difference in rates observed do not appear to be significantly different from those containing other C' reagents.

IV. The Role of the Properdin System in Phagocytosis and Cytopepsis

Although the data of prior experiments suggest that heat labile factors were responsible for enhanced rates of cytopepsis of H. capsulatum by mouse macrophages, the factors involved are not definitely shown. The possibility exists that instead of the participation of C' in digestion, properdin may be the factor involved, or perhaps the properdin system (i.e. properdin, C' and Mg^{++}) may be functioning. The following experiments were carried out in an attempt to define the relative importance of these factors in phagocytosis and cytopepsis.

Peritoneal exudates were obtained from groups of normal mice and mice immunized with H. capsulatum. The peritoneal phagocytes were lightly centrifuged and washed once with cell maintenance fluid (CMF). The macrophages were resuspended in CMF, counted in a hemocytometer and the suspension adjusted to the desired concentration. Twenty five million "immune" phagocytes in a volume of 20 ml CMF were added to each

of five 50 ml glass-stoppered flasks and two million cells in a volume of 1.25 ml CMF added to each of several cover slip containing planchets. This procedure was then repeated with phagocytes from normal animals.

After overnight incubation, during which time the cells attached to the glass, the supernatant fluids were removed, the attached cells washed once with CMF, and once with Earle's BSS. Next, additions were made to the flasks and cover slip preparations with suspensions containing 1 x 10^5 P-32 labeled H. capsulatum per milliliter as outlined in Table 14.

Properdin activity in the final solution before addition of organisms (Flasks 2N and 2I) was 6 to 8 units/ml as determined by the zymosan assay (Pillemer et al., 1956), while activity after heating (Flasks 3N and 3I) was undetectable. C' activity in the absence of properdin (Flasks 4N and 4I) was found to be 50 units/ml before addition of yeast cells and after addition of properdin (Flasks 5N and 5I) was 5 units/ml as determined by the micro-Kolmer assay (Kolmer, Spaulding and Robinson, 1951).

Immediately following addition of the cell suspensions to the respective flasks (Table 14), supernatant samples were taken for radioassay, as were cover slip preparations. Phagocytosis was allowed to proceed for 4 hours with slow shaking. Samples were removed at the end of 2 and 4 hours.

After the 4th hour sampling, the supernatant fluids were removed, and the attached macrophages containing ingested P-32 labeled H. capsulatum were washed once in CMF and once in Earle's BSS. Menstruums of

similar composition previously contained in each flask or cover slip preparation were added back and the digestion period followed for 54 hours while incubating at 35.5°C without agitation, except just prior to sampling. Figures 30 through 36 show the results of this experiment.

TABLE 14

Flask and cover slip contents for determination of the effect of partially purified calf properdin (PPCP) and C' on phagocytosis and digestion by normal and "immune" mouse phagocytes.

Flask or Cover slip	Menstruum***	Total Volume/flask ml
1N* & 1I* flasks	CMF	20
2N & 2I flasks	Incomplete CMF,** 10% PPCP, 10% calf serum	20
3N & 3I flasks	Incomplete CMF, 10% heated PPCP, 10% calf serum	20
4N & 4I flasks	Incomplete CMF, 10% C', 10% calf serum	20
5N & 5I flasks	Incomplete CMF, 10% C', 10% PPCP	20
N & I cover slips	CMF	1.25

* N indicates normal phagocyte-containing flask; I indicates "immune" phagocyte-containing flask.

** 70% Earle's BSS, 10% lactalbumin hydrolysate solution.

*** Each menstruum contained 1 x 10^5 P-32 labeled H. capsulatum per milliliter.

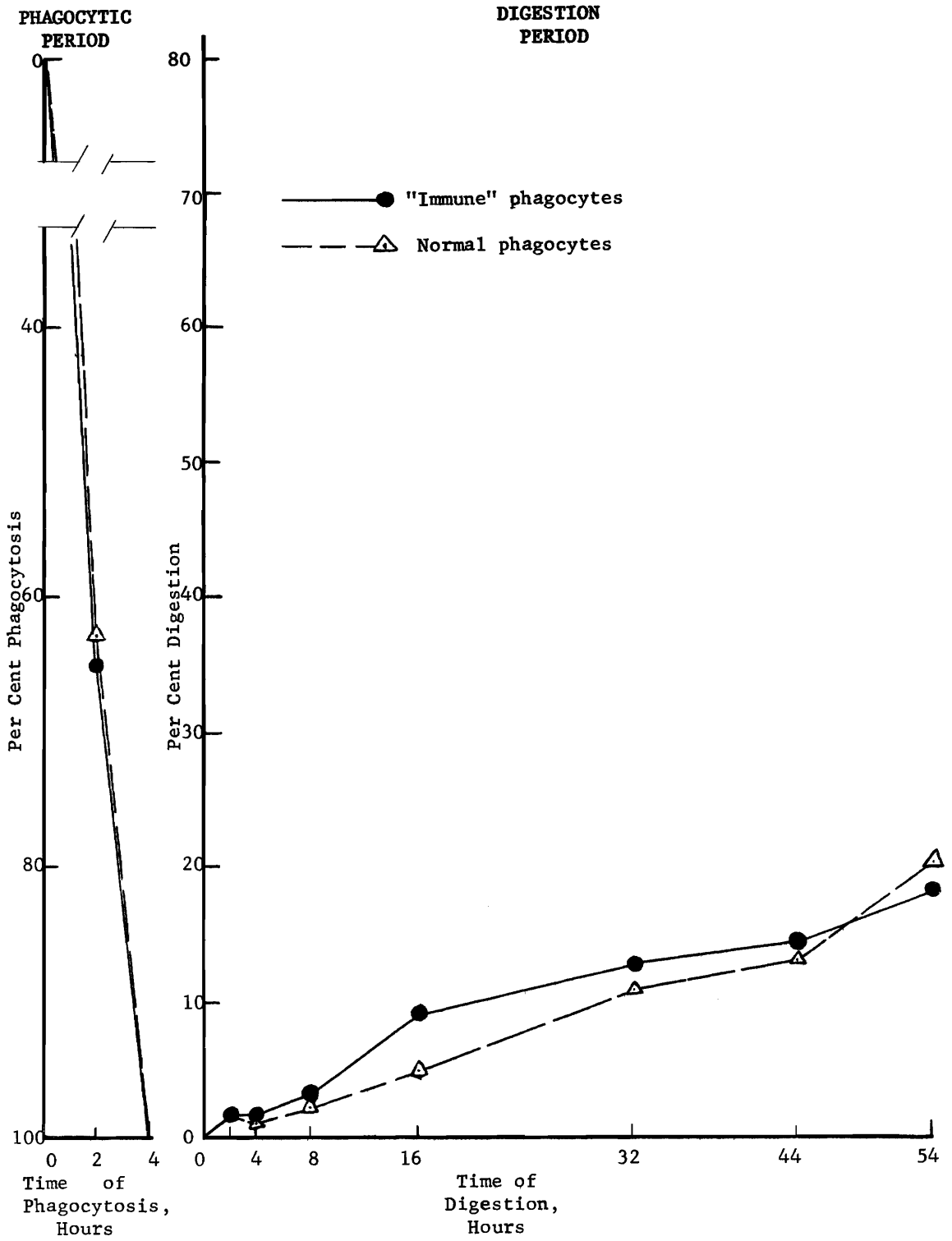

Figure 30. Phagocytosis and digestion of H. capsulatum by "immune" and normal mouse peritoneal macrophages in the presence of cell maintenance fluid.

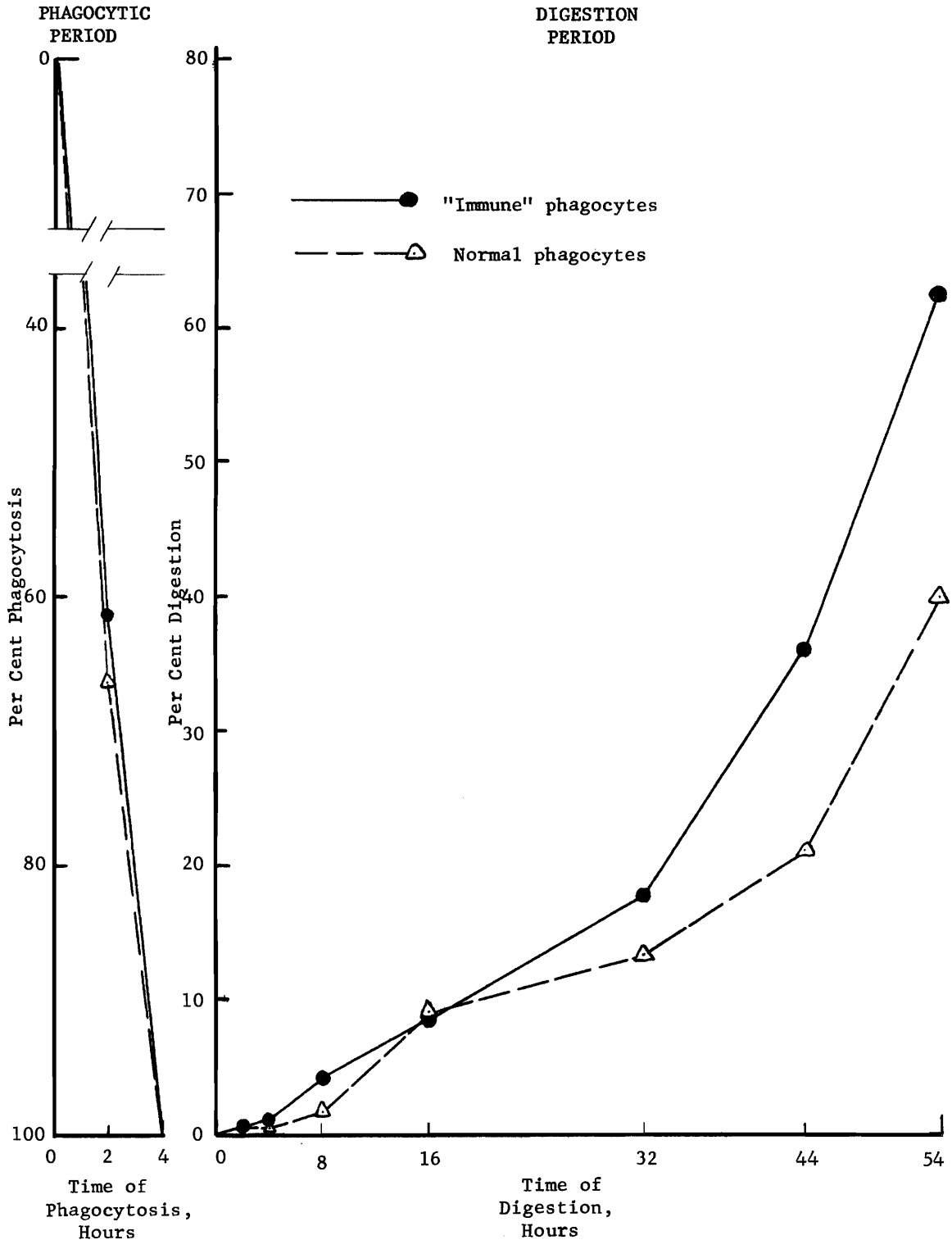

Figure 31. Phagocytosis and digestion of H. capsulatum by "immune" and normal mouse peritoneal macrophages in the presence of cell maintenance fluid containing 8 units/ml partially purified calf properdin.

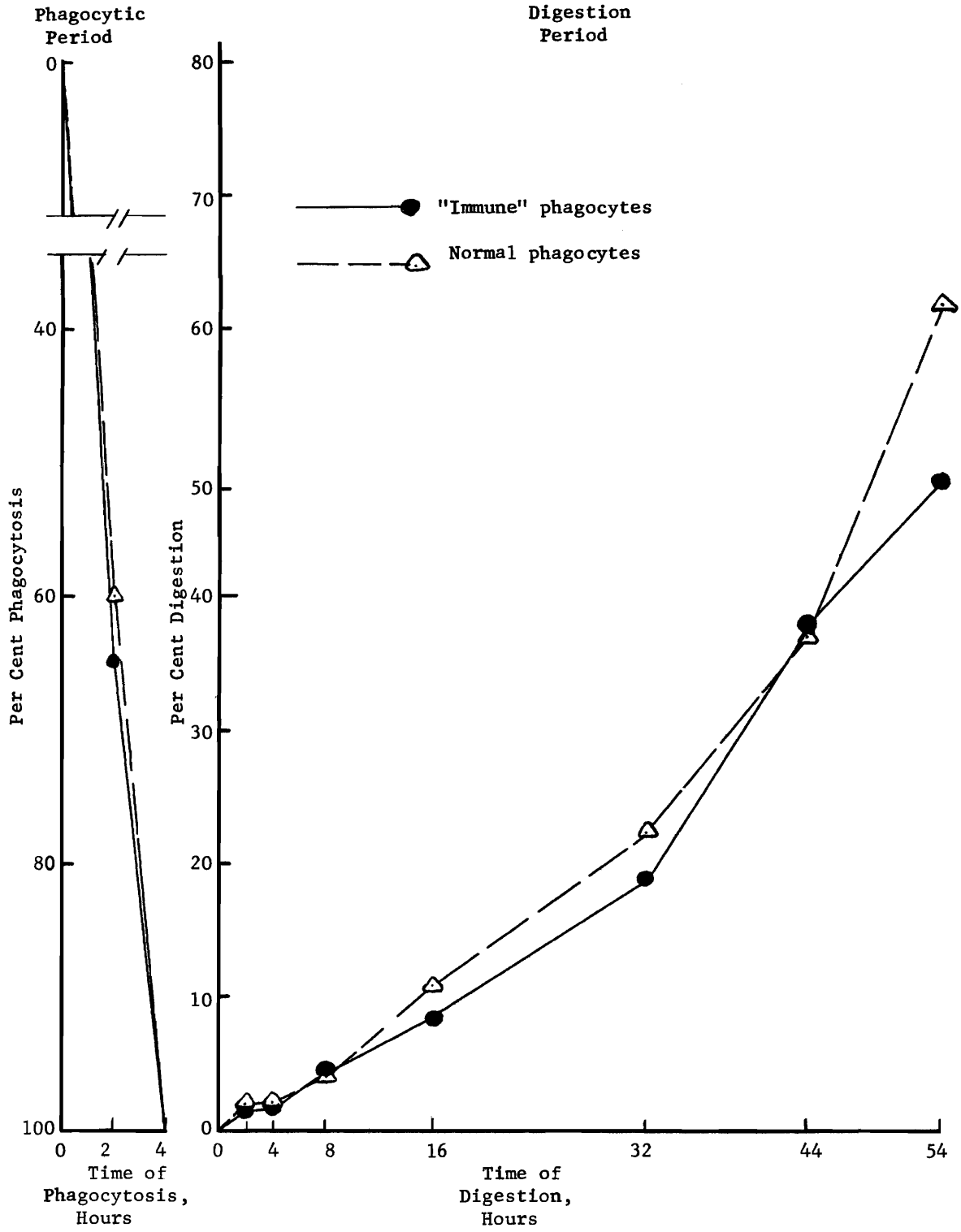

Figure 32. Phagocytosis and digestion of H. capsulatum by "immune" and normal mouse peritoneal macrophages in the presence of cell maintenance fluid containing heat inactivated partially purified calf properdin.

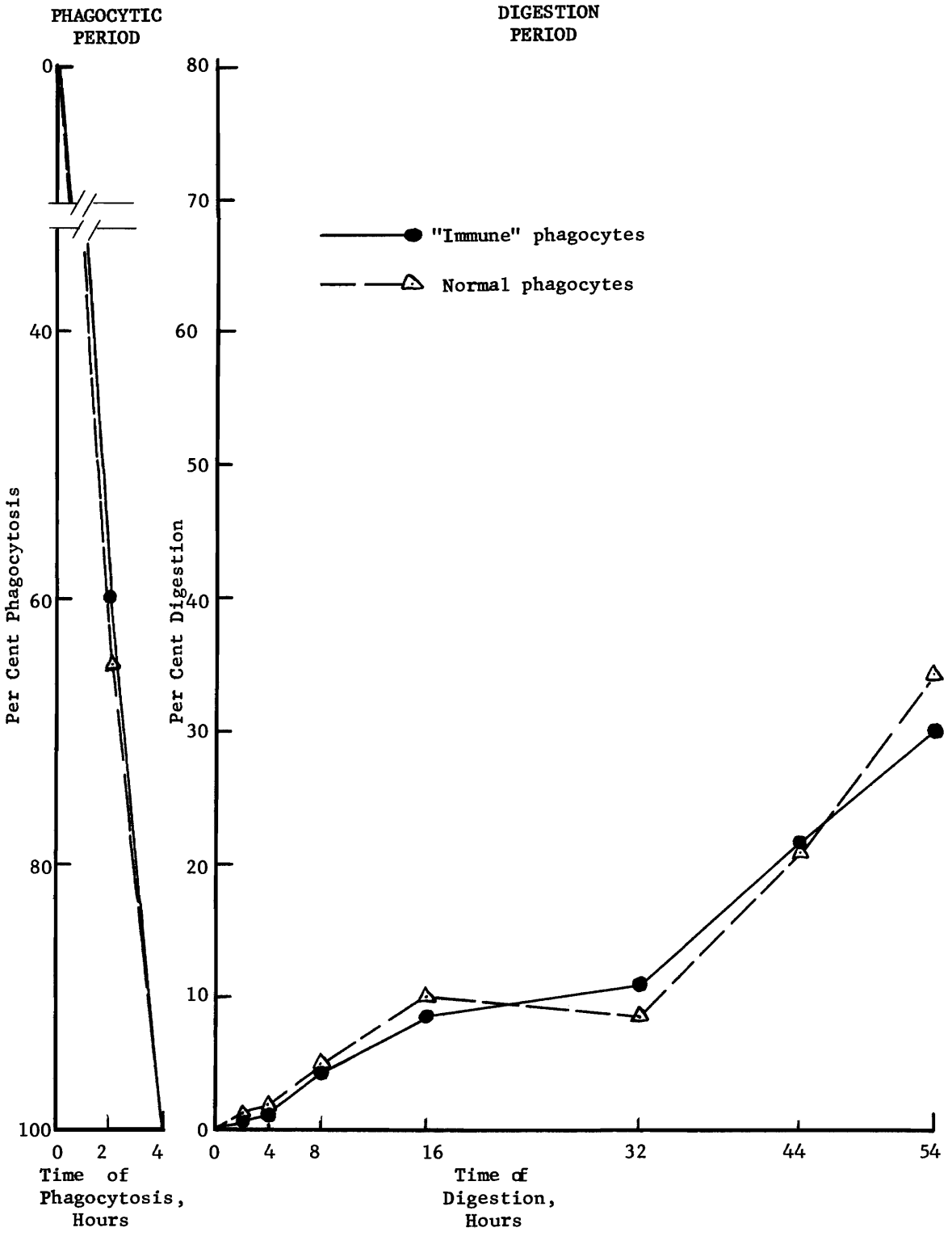

Figure 33. Phagocytosis and digestion of H. capsulatum by "immune" and normal mouse peritoneal macrophages in the presence of cell maintenance fluid containing 50 units/ml guinea pig complement.

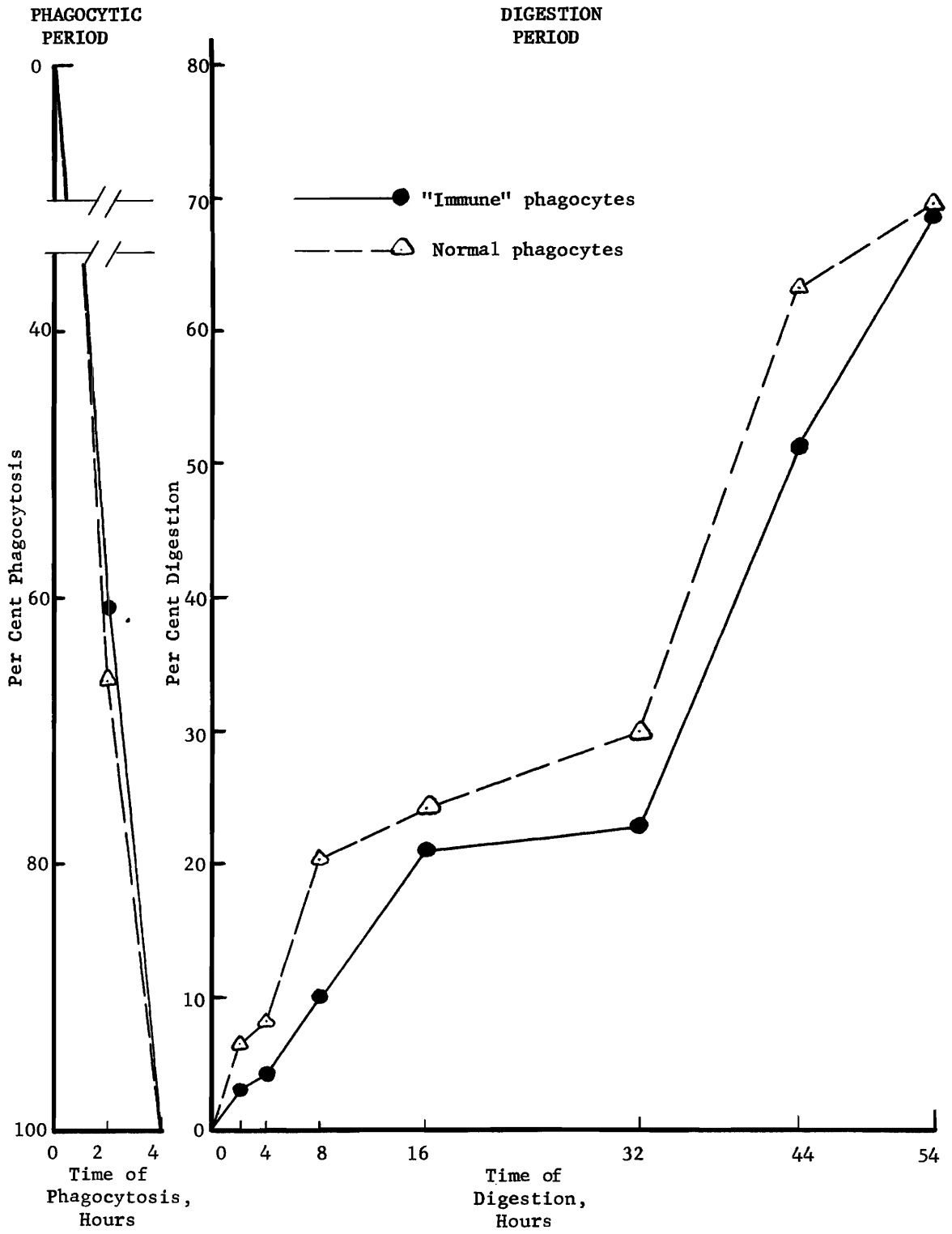

Figure 34. Phagocytosis and digestion of H. capsulatum by "immune" and normal mouse peritoneal macrophages in the presence of cell maintenance fluid containing guinea pig C' and partially purified calf properdin.

A. Phagocytosis.

The first five figures show the comparative rates of phagocytosis and digestion of H. capsulatum by "immune" and normal mouse macrophages in the presence of different menstruums, i.e. CMF, medium containing partially purified calf properdin (PPCP) etc. These figures are based upon the radioactivity in the supernatant medium as a function of time and are expressed as per cent ingestion or digestion as determined by previously described methods.

The rate of phagocytosis in the presence of C' and/or PPCP was not significantly different from that given in the presence of CMF where these factors were absent. Inspection of the phagocytic portions of Figures 30 through 34 shows that the amount of ingestion that had taken place two hours after addition of organisms to the system varied from approximately 60 to 66% of the total number ingested. This is based upon loss of radioactivity from the supernatant medium. Likewise, the rates of phagocytosis by "immune" and normal cells were comparable in all cases.

B. Digestion.

Comparisons of "immune" and normal cells during the period of digestion, shown in Figures 30 through 34, do not appear to be significantly different except in the presence of PPCP alone, where the "immune" cells were much more active than normal cells (Figure 31). A tendency toward this increased activity was also found in the presence of CMF (Figure 30), an observation substantiated by microscopic observations of cover slip preparations. However, in the presence of heated PPCP (Figure 32),

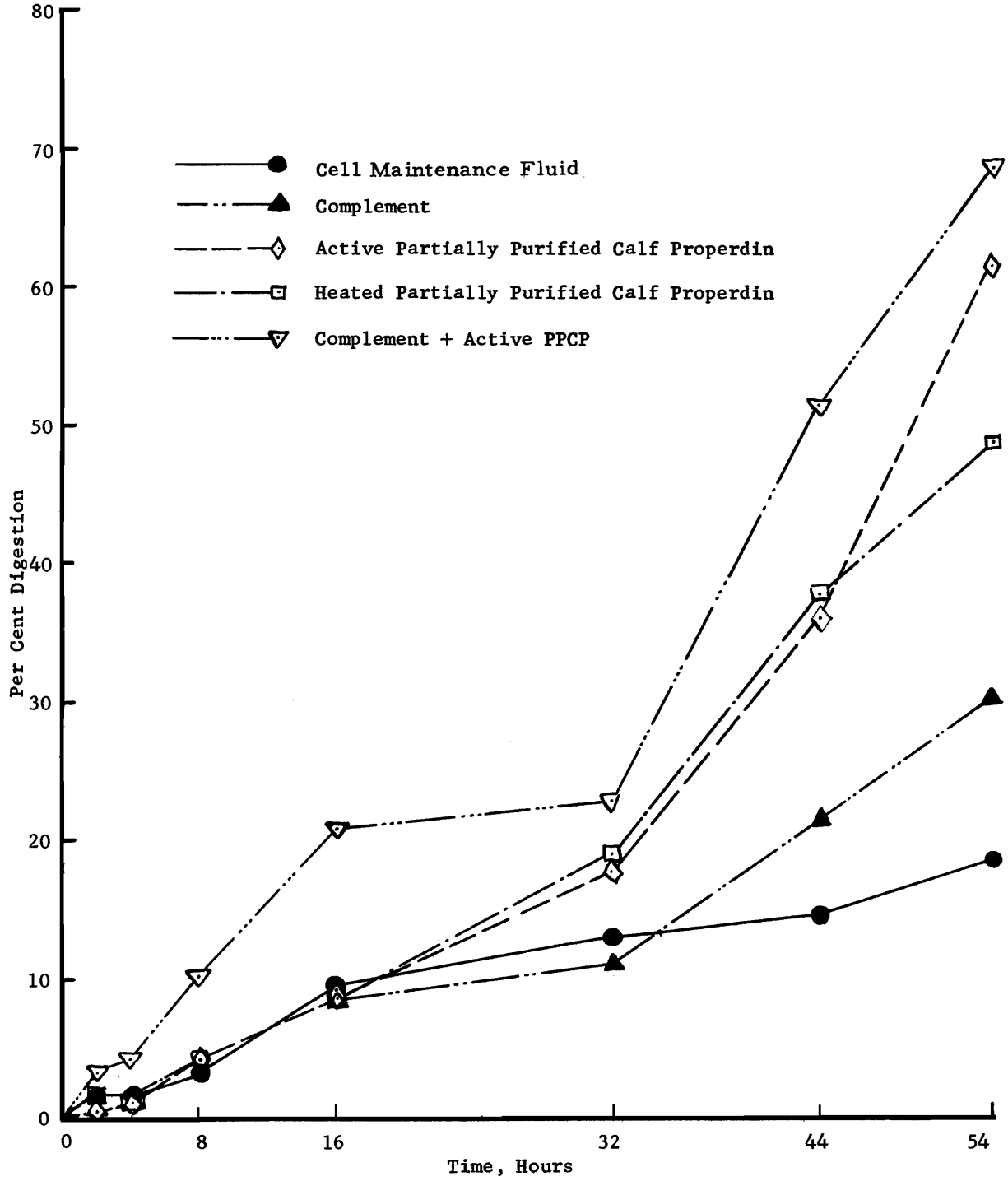

Figure 35. Digestion of H. capsulatum by "immune" mouse macrophages in the absence and presence of complement and/or partially purified calf properdin.

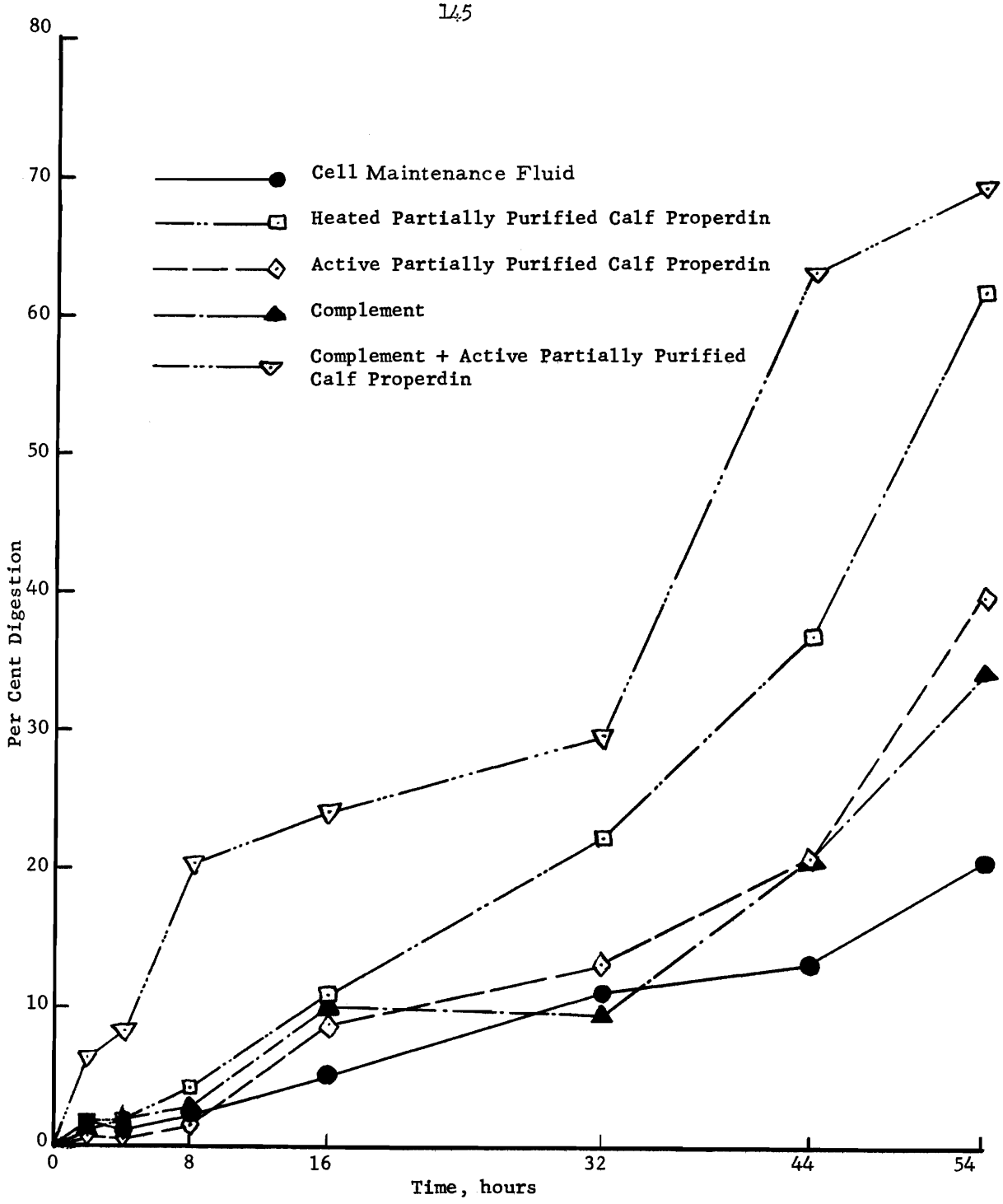

Figure 36. Phagocytosis and digestion of H. capsulatum by normal mouse macrophages in the presence and absence of C' and/or partially purified calf properdin.

complement alone, (Figure 33), or C' with PPCP (Figure 34) no significant differences between the two cell populations were found.

In comparing the effects of the various menstruums on the rate of cytopepsis within the "immune" and normal cell populations certain significant differences were noted. Inspection of Figure 35 shows that the "immune" cells were only slightly active in digestion in the presence of CMF. (Note that the phagocytic period has been deleted from Figure 35 and 36 because of comparable ingestion rates in the various menstruums as previously shown). C' gave enhancement of cytopepsis over CMF, with increases showing in 44 hours. However, both unheated and heated calf properdin caused increased rates of digestion over that given by both CMF and C', with differences appearing in 32 hours. The presence of both C' and PPCP in the menstruum caused the highest rates of digestion. The increased effect appearing within two hours after the digestion period began.

A plot of normal cell activity (Figure 36) in the presence of the various menstruums gives a similar picture as for "immune" cells with enhanced cytopeptic rates over that found in CMF appearing in the presence of C', PPCP, heated PPP, and combined C' and PPCP. Again, the most active cytopepsis was given when both properdin and C' were present.

In these data, however, an unexplained anomaly appeared. This is the increased rate of digestion found in the presence of heated PPCP over that of the unheated material. At present, a satisfactory explanation for this phenomenon cannot be given.

DISCUSSION

The experimental results indicate that under the conditions employed macrophages from normal and immune mice were equally capable of phagocytizing P-32 labeled Histoplasma capsulatum. This was found in both the fixed phagocyte system (Method I) where macrophages were maintained attached to glass, and in the "free" phagocyte system (Method II) where the macrophages were allowed to remain freely suspended during the incubation period. Also, this relationship was noted in the presence of both heated and unheated homologous and heterologous sera; in the presence of specific antibody with and without complement; in the presence of C' reagents using normal phagocytes; and in the presence and absence of the properdin system or its separate components.

The finding that normal and "immune" macrophages do not differ in their rates of phagocytosis confirms that of previous investigators (Hill and Marcus, 1960, and Miya and Marcus, 1961). However, the fact that heat labile factors of serum did not enhance ingestive rates is in conflict with the findings of many investigators. For example, Miya and Marcus (1961) reported that increasing amounts of C' caused increased rates of ingestion of H. capsulatum by normal and "immune" macrophages. However, in their investigation C' was the sole source of protein in the culture medium employed and it is possible that increasing phagocytic rates reflected the larger amounts of protein in the medium, thereby

giving a more desirable environment for ingestion at higher C' concentrations.

Other investigators, many of whom have employed bacterial agents in their models, have reported enhanced phagocytosis in the presence of complement. In this regard, the study of Ward and Enders remains outstanding. These workers were able to show that C' brought about an increased rate of ingestion of pneumococci by phagocytes in the presence of antibody, but did not increase the total number of organisms ingested when compared to the use of antibody alone. This finding, along with results of others (e.g., Wright and Douglas, 1903; Moore, 1919) shows the importance of C' in the phagocytosis of bacteria.

The experimental evidence found in this study concerning the role of the properdin system in the ingestion process is in agreement with evidence presented by Rutenburg and Fine (1957) and Miya and Marcus (1961) but conflicts with that of Nanni (1958). It is of interest to note, however, that in the latter case, complement was not required to give enhanced rates of phagocytosis in the presence of properdin and that the addition of Mg^{++} in normal serum gave high rates of ingestion. This may suggest that since Mg^{++} was used in similar concentrations in the two cases increased rates of phagocytosis could have been due to as yet obscure and unspecified conditions.

The influence of antibody in phagocytosis as noted in this study is different from that usually reported; it is generally accepted that antibody opsonizes bacteria such that they may be ingested more readily. This relationship is most readily demonstrated with organisms in their

smooth phase of growth, and may not appear when the bacteria are in the rough phase. This fact suggests a reason why antibody is ineffective in opsonizing H. capsulatum. That is, the mycotic agent may have a sufficiently rough cell wall to eliminate the requirement for opsonization by specific antibody.

Another explanation for the results may also be given. The fact that the phagocytic rates of both cell populations were nearly alike in this study under all conditions employed and from past evidence showing similar findings with nonhumoral factors (Miya and Marcus, 1961) it appears likely that under the circumstances being employed the in vitro uptake of H. capsulatum may be regulated by physical factors, such as size of the organisms, rather than serum factors. It could then be because of these physical factors that any effects brought about by heat labile factors of unheated sera were masked.

Results concerning cytopepsis bring out several interesting observations. For example, employing the system where phagocytes were allowed to remain in suspension during incubation (Method II) digestion of H. capsulatum by "immune" cells was observed to be generally more rapid than that of normal cells, confirming the results of Hill and Marcus (1960) and Miya and Marcus (1961). One notable exception was observed with heated calf serum. The results with this reagent correspond with data obtained employing Method I where cytopeptic rates were measured after the macrophages had become attached to glass. Hill and Marcus (1960), however, were able to show higher rates of digestion by "immune" cells over normal cells in the presence of heated calf serum,

also using H. capsulatum as the indicator organism. On the other hand, Miya and Marcus (1961) reported the necessity of having heat labile factors for enhanced digestion by "immune" phagocytes when rabbit or guinea pig sera were used. This limiting factor was not found here using the "free phagocyte system" since "immune" cells were more active in either heated or unheated sera, except in the presence of heated calf serum. However, the differences in results of Miya and Marcus (1961) and those reported here are not absolute, since, as will be discussed below, heat labile factors did enhance cytopepsis.

It should be pointed out that the findings that "immune" macrophages were more cytopeptic than those from normal animals using the free phagocyte procedure are at variance with those of experiments employing macrophages attached to glass, where no differences between cell populations were found by the isotope assay of supernatant fluids. The conflicting results are probably due to differences in techniques employed, although there is no obvious reason why the former procedure should be more sensitive than the latter. However, one explanation might be that the phagocytes were more active in the free phagocyte system due to their earlier infection with H. capsulatum after being obtained from animals, thus giving the observed results.

Comparison of effects of homologous and heterologous sera on cytopepsis by mouse macrophages reveal several interesting points. First, the most rapid rates of digestion by both cell populations were obtained in the presence of homologous serum and guinea pig serum. The results with mouse serum might be expected since it would probably provide factors

required by mouse phagocytes at optimal concentrations, although McElree and Downs (1961) did not find this to be the case with a rat phagocyte-rat serum system where the serum was actually toxic for the macrophages. However, the mechanism of enhanced cytopepsis by guinea pig serum cannot easily be rationalized, except perhaps on the basis of its high C' level, although the use of heated serum from this source also gave levels of digestion above human, rabbit or calf serum. It is worth recalling here that McElree and Downs (1961) obtained similar enhancement in phagocytosis of Pasteurella tularensis in the presence of guinea pig serum by rat phagocytes.

Another observation with regard to effects of homologous and heterologous sera was that human, calf and rabbit sera all gave similar rates of digestion by both normal and "immune" phagocytes. In the presence of unheated calf or rabbit serum cytopeptic rates were generally higher than with human serum. Indeed, the presence of heat labile factors (C' and/or properdin) generally brought about increased rates of digestion of the yeast phase mycotic agent by a cell population. An exception to these results was obtained when human serum was used. Significant enhancement was not found with this unheated serum. Since this latter result was noted in both cell populations it is quite possible that a system of mouse phagocytes and human serum was not compatible for maximum digestion by cells.

In further investigation of the role of C' and antibody using the fixed phagocyte system, complement was found to enhance the digestive capacity of both normal and "immune" macrophages and this was amplified

in the presence of the heat stable factor. Antibody alone also gave slight enhancement of cytopepsis, but not nearly to the degree found by the presence of C'. Again Miya and Marcus (1961) made similar observations concerning C' action by determining the effect of heated and unheated normal and immune serum from rabbits. Antibody in this study, however, was reported to effect no change in the rate of cytopepsis over its absence.

In the studies involving the role of C' and antibody in cytopepsis by normal and "immune" phagocytes a direct comparison between rates of cytopepsis of the two cell populations could not be made because of the increased rates of normal cell destruction due to H. capsulatum infection. However, the fact that the normal cells did deteriorate so rapidly further emphasizes the difference in resistance between the two cell populations, as revealed in the data of other investigators (e.g., Lurie, 1942; Suter, 1953; Hill and Marcus, 1960).

This difference was further shown by microscopic observations of cover slip preparations containing normal and "immune" cells. By this procedure the "immune" cells were found to carry out digestion more rapidly in the absence of heat labile substances or antibody than normal cells. This observation is in agreement with those reported by Donaldson et al. (1956) and Hill and Marcus (1960).

Despite the finding that C' may lead to enhanced rates of cytopepsis, the C' components involved in this phenomenon remain unknown. Several explanations may be offered for the lack of change of digestive rates in the absence of one or more C' components. First, the possibility

exists that deleted components were not completely eliminated, leaving small amounts available to take part in cytopepsis. This is, however, unlikely in the cases of endpiece and R3 where the lack of hemolysis of sheep red cells in the standard micro-Kolmer titration showed the presence of less than 1 unit/ml of C'2 and C'3 respectively. The lower limit of the midpiece and R4 titrations was 10 units/ml, therefore, it may be that sufficient C'1 in the M and C'2 or C'4 in the R4 was present to provide enhanced cytopepsis. It follows that it may be definitely stated only that the lack of C'2 or C'3 from guinea pig serum did not reduce the rate of digestion given by whole C'.

Another explanation for the observed results could be that the factors causing enhanced cytopepsis may not be associated with any one component of C' but may be a characteristic of two or more components. Therefore, if both C'1 and C'2, for example, had the capacity to increase rates of digestion; elimination of one of these factors would cause little change over that found when both were present.

A third possibility also exists that increased cytopepsis is not associated with the hemolytic components of C' at all, but rather with a separate heat labile factor in guinea pig serum. Furthermore, this factor may not be separable into fractions as can be done with C'. Such a possibility has been expressed in the case of opsonins by Ecker, Pillemer and Kuehn (1942) who found no relationship between the hemolytic and opsonic properties of guinea pig serum (as well as other sera from lower animals) toward staphylococci. However, the opsonin that was present was heat labile, being inactivated by the same temperature treatment

as C'. Also, Tullis and Surgenor (1956) maintain that phagocytosis promoting factors (PPF) do not include C', even though PPF can be removed by heating at 56°C for 20 minutes.

The influence of heat labile factors in enhancing cytopepsis are evident throughout the data. However, the exact factor(s) involved are not defined. That is, the relative effects of C', properdin, or the properdin system involved in this phenomenon are unknown. In experiments to define the role of each of these factors it was found that both heated and unheated properdin caused enhanced rates of digestion by the monocytic cells from normal and immune mice, even though complement was absent. This would indicate that heat stable globulins can give increased rates of digestion, and that properdin is probably not involved in the enhancement noted here. This reasoning is more plausible when one considers that C' is required for properdin action in bactericidal, virus neutralizing, and certain hemolytic activities of serum (Pillemer et al., 1954). The fact that increased digestion was found in the presence of heated properdin, where properdin activity was less than 1 unit/ml by zymosan assay, and that C' was also absent indicates that this enhancement was due to factors other than the properdin system.

Activation of the properdin system was brought about by the addition of C' and properdin to the same medium. It was in this medium that the most active digestion took place by both normal and "immune" macrophages. This action is attributed to the properdin system, suggesting that it is intimately involved in cellular resistance of the host.

SUMMARY

Phagocytosis and cytopepsis of P-32 labeled Histoplasma capsulatum by normal and "immune" mouse macrophages were studied in the presence of various humoral factors. The results of this investigation were as follows:

1. Phagocytic rates of the organisms by macrophages from normal and immune mice were not significantly different in the presence of heated or unheated homologous and heterologous sera.

2. Using a system of suspended phagocytes with the mycotic agent, macrophages from immunized animals digested the organism more rapidly than did those from normal mice in the presence of heated and unheated homologous and heterologous sera, except in the presence of heated calf serum. In a system where phagocytes were first allowed to attach to glass before infection differences between the two phagocyte populations were not noted by supernatant P-32 activity. However, the enhanced capacity of the "immune" cells was noted by other means of measurement in this latter system.

3. Cytopeptic rates for both normal and "immune" mouse phagocytes were highest in the presence of heated and unheated mouse or guinea pig serum, while rates in the presence of heated human, rabbit or calf serum were least and not significantly different. Unheated rabbit and calf serum induced intermediate rates of digestion.

4. C' enhanced digestion of H. capsulatum by normal and "immune"

macrophages. Rabbit (heterologous) antibody against H. capsulatum enhanced digestion, but much less so than did heterologous (guinea pig) C'. Antibody and C' together yielded a more rapid rate of cytopepsis than did either factor alone.

5. The presence of guinea pig C' reagents lacking one or more C' components did not alter the rate of ingestion or digestion of H. capsulatum by normal mouse peritoneal phagocytes over that given by whole C'.

6. Heated and unheated partially purified calf properdin (PPCP) caused enhanced rates of cytopepsis by both "immune" and normal phagocytes; the effect obtained with heated and unheated properdin was greater than that with C'. The results with the heated and unheated PPCP were apparently due to factors other than properdin.

7. The presence of complement and properdin in the same medium resulted in the most rapid rates of cytopepsis. This effect has been attributed to the properdin system on the basis of the evidence presented here.

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THE ROLE OF HUMORAL FACTORS IN CELLULAR RESISTANCE

by

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ABSTRACT

In vitro experiments were undertaken to determine the role of complement, properdin and antibody in the phagocytosis and cytopepsis of Histoplasma capsulatum by peritoneal macrophages from normal and immunized mice. Two methods of approach were employed. The first involved the use of freely suspended infected macrophages while the second made use of macrophages attached to glass before infection with the organism. Phagocytic rates were measured by microscopic examination of stained smears in the former method, while supernatant sampling techniques for P-32 activity was employed in the second method. Both procedures involved sampling the medium during incubation to follow cytopepsis. The hypothesis involved was that the P-32 activity appearing in the medium with time was a function of cytopeptic activity of the macrophages on ingested P-32 labeled organisms.

Under the experimental conditions it was found that rates of phagocytosis were not altered by the presence of various humoral factors studied nor were any differences noted in the rates of ingestion by normal and "immune" populations of cells.

The rates of cytopepsis, however, were definitely enhanced by the presence of heat labile factors (i.e., complement and the properdin system, which consists of complement, properdin and Mg^{++}) and less so by antibody, although complement and specific antibody produced increased cytopeptic rates. Studies using complement reagents lacking one or more components failed to reveal which fractions of the heat labile factor might be responsible for enhanced cytopepsis.

The "immune" population of cells was found to have increased cytopeptic activity over normal cells in a freely suspended phagocyte system, but not by a fixed macrophage system. This finding points to the importance of the technology involved in biological investigative models.

RESEARCH PROPOSALS

submitted

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RESEARCH PROPOSALS

1. The evidence that cellular immunity exists is abundant. However, the possible role of either absorbed exogenous or preformed endogenous antibody remains to be determined. A definitive investigation of this intracellular factor should be conducted.

2. The importance of cellular immunity in viral diseases is largely unknown, especially in the final disposition of the agents (i.e. cytopepsis). A study of this problem would aid in elucidating mechanisms of resistance to viral agents.

3. The enhancement of phagocytosis and cytopepsis by the presence of complement is evident. It is possible that only one or two of the components of this factor may be required for these cellular processes. Inquiry into the role of each of the C' components alone and in combination with each other, therefore, is indicated.

4. The hypothesis is offered that an interaction exists between chemotherapeutic agents, as well as other drugs, and phagocytic cells of the host. That is, certain drugs may aid in the disposition of an infectious agent while others may hinder this process. In vitro investigations on host cell-drug interactions should be carried out and attempts made to correlate these with known in vivo effects.

5. Evidence concerning increased cellular participation in phagocytosis and cytopepsis following immunization is largely based on increased

function. The basic metabolic alterations of macrophages attributable to immunization require investigation.

6. Correlation of results in phagocytosis and cytopepsis by macrophages maintained in vitro with similar phagocytes in situ are lacking. Experiments designed to give this information are needed.

7. Bacterial endotoxins are known to nonspecifically increase host resistance to infection but little is known concerning the mechanism of action involved. What influence does the lipopolysaccharide have on humoral and cellular functions which lead to increased resistance? The hypothesis is offered that one effect is to increase metabolic rate of the macrophages of the endotoxin-treated animal.

8. The properdin system was found to increase cytopeptic activity of both normal and "immune" macrophages. Further investigations are required to determine whether this complex of properdin, complement and Mg^{++} act on the phagocyte or on the disease agent. Furthermore, the mechanism of action of the properdin system on the phagocyte or disease agent requires elucidation.

9. Present knowledge concerning the action of complement has been derived largely through study of erythrocyte lysis. The relationships that these findings have with the participation of C' in opsonization, which are tacitly assumed, should be stated and tested.

10. In recent years the identity of properdin has been cast in doubt due to its similarity of action to antibody. An investigation leading to definitive identification of these humoral factors is required.

Furthermore, it is noted that certain blood dyscrasias (e.g. macroglobulinemia of Waldenstrom) are characterized by the presence of high molecular weight globulins. It is proposed that such globulins may exhibit properties similar to properdin. Inquiry into these characteristics should be carried out.